Formation of neural and epidermal lineages during the differentiation of human embryonal carcinomacells

Christie, Victoria Buyers

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Victoria Buyers Christie

Within the ectoderm of the developing human embryo, cell fate determination is largely modulated by factors activated by cell-to-cell signalling. In vertebrates, ectoderm primarily differentiates into neural cells that make up the central nervous system and lining epithelium, commonly known as the skin epidermis. One of the main regulators of this process is the bone morphogenetic protein (BMP) signalling pathway. While this pathway, and its involvement in ectodermal patterning, has been well studied in lower vertebrates there is little data available for its role in human ectodermal development. The main aim of this investigation was to establish whether the human TERA2.SP12 embryonal carcinoma (EC) stem cell line may be a viable model useful in the study of this particular mechanism in humans. Pluripotent human embryonal carcinoma cells have already been shown to represent a researchable model of human embryogenesis. It has also been shown that the differentiation of the human EC cell lines can be induced in a controlled manner using certain reagents such as retinoic acid and hexamethylene bisacetamide (HMBA). In this study it has been determined that the TERA2 EC cell sub-line TERA2.SP12 can also be induced to differentiate in a controlled manner. Retinoic acid induces cultured TERA2.SP12 cells to differentiate into a variety of cell types, including neurons and glial cells. Using molecular techniques such as flow cytometry, western analysis, immunocytochemistry and RT-PCR these cultures were seen to express a range of proteins associated with the neural lineage. Non-neural cell types are also found in these cultures that are morphologically similar to those found when TERA2.SP12 EC cells are exposed to HMBA. Differentiation of TERA2.SP12 EC cells induced by HMBA was shown to differ to that induced by retinoic acid in that no morphologically identifiable neurons are observed in cultures grown as adherent monolayers. Through analysis of specific proteins it was noted that the expression of neural markers was significantly reduced. Interestingly, cells exposed to HMBA were found to regulate significantly higher levels of certain extra-cellular proteins such as fibronectin, laminin and cytokeratin-8. One of the mechanisms that these molecules have been previously associated with is the development of epidermis. Proteins and genes present in the BMP signalling pathway have also found to be expressed by these TERA2.SP12 differentiating cultures. These results indicate that retinoic acid induces the formation of neural tissues as well as non-neural tissues from human EC cells. Exposure to HMBA, however does not result in terminally differentiated neural phenotypes but induces the formation of a different lineage expressing high levels of markers that could be associated with developing epithelia such as immature epidermis. Accordingly, I conclude that the TERA2.SP12 EC cell system may be utilised as a potential model for cell fate specification between neural and non-neural lineages in man.

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Formation of Neural and Epidermal Lineages During the Differentiation of Human Embryonal Carcinoma Cells

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MSc Thesis

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Publications

Formation of neural and non-neural lineages during the differentiation of human embryonal carcinoma cells.

Christie V.B, Przyborski S.A.

in preparation

Manipulation of human pluripotent embryonal carcinoma stem cells and the development of neural subtypes.

Stewart R, **Christie V.B**, Przyborski S.A.

Stem Cells, *in press*

Abstracts

Cell fate determination in the human embryonic ectoderm and the induction of the neural lineage.

Horrocks G.M, **Christie V.B**, Lauder L, Hayman M.W, Przyborski S.A.

Abstract from April 2003 meeting of BNA, Harrogate, UK.

Characterisation of neural and non-neural pathways of differentiation by human pluripotent stem cells in vitro.

Christie V.B, Przyborski S.A.

Abstract from December 2002 meeting of Neuroscience North East, Sunderland, UK.

Glossary

- EC cell — Embryonal carcinoma cell
- ES cell — Embryonic stem cell
- GCT — Germ cell tumour
- ICM — Inner cell mass
- DMSO — Dimethylsuloxide
- RA — Retinoic acid
- HMBA — Hexamethylene bisacetamide
- SSEA-1 — Stage specific embryonic antigen-1
- SSEA-3 — Stage specific embryonic antigen-3
- SSEA-4 — Stage specific embryonic antigen-4
- BUdR — 5-bromouracil 2'deoxyribose
- IUdR — 5-iodouracil 2'deoxyribose
- DMEM — Dulbecco's modified eagles medium
- PBS — Phosphate buffered saline
- TBS — Tris buffered saline
- BSA — Bovine serum albumin
- rpm — Rotations per minute
- UV — Ultra violet
- PFA — Paraformaldehyde
- BMP — Bone morphogenetic protein

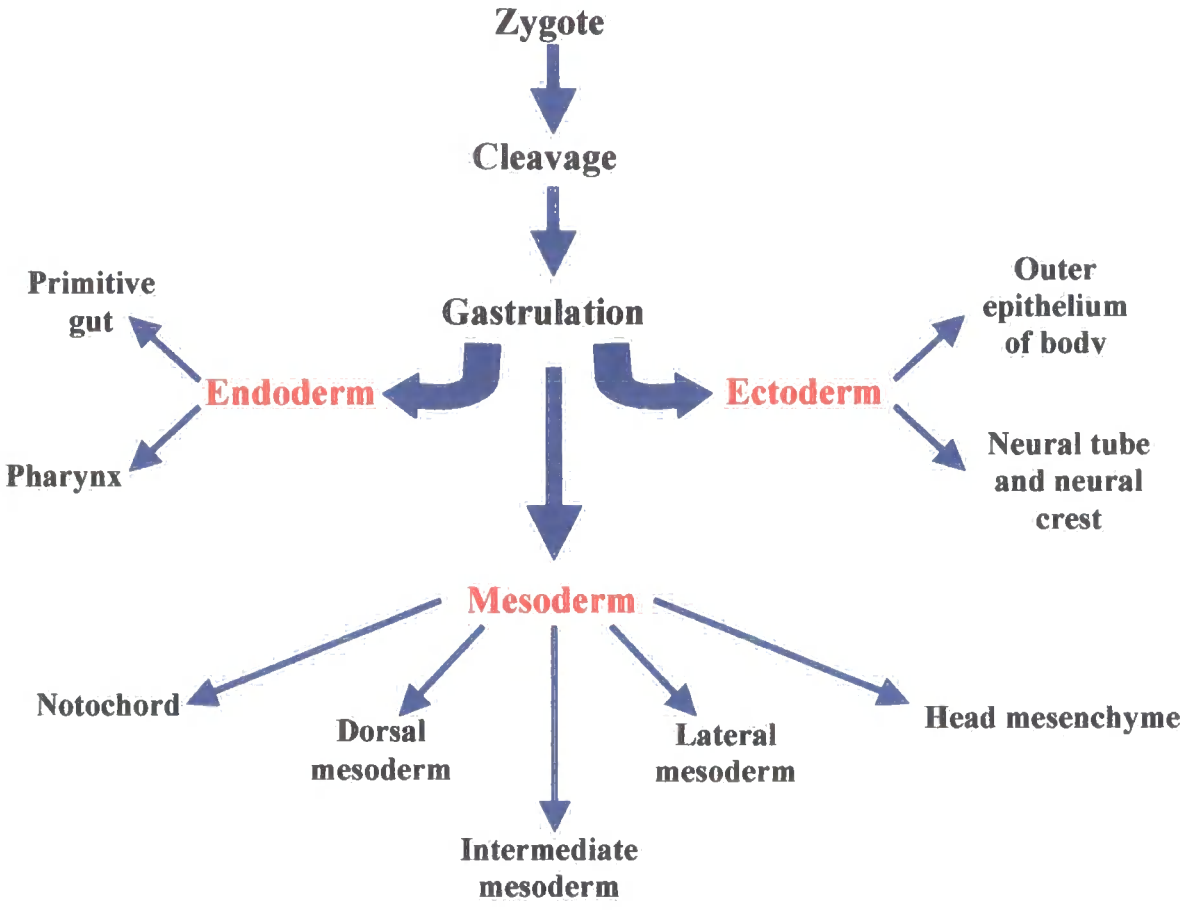
Chapter 1

Introduction

1.1 Cell Fate Determination in the Embryonic Ectoderm:

After a zygote is fertilized the cells within it start to divide and eventually begin to organize themselves into a structured mass containing three distinct layers of cells, termed the germ layers. This process is called gastrulation and begins on day 14, ending on day 19 of human embryonic development. These germ layers are called the endoderm, mesoderm and ectoderm they are derived from the epiblast and they give rise to tissues of the embryo (Figure 1.1). In brief, the ectoderm develops into the outer epithelia and nervous system, the endoderm forms the epithelial linings of the respiratory and digestive tracts; including the glandular cells of the liver and pancreas, and the mesoderm forms the smooth muscular coats, connective tissue, skeleton, striated muscle, reproductive and excretory organs, the vessels supplying these organs, and blood cells and the bone marrow. Cells within the germ layers have the ability to differentiate into terminal end products and migrate away from the layers, but are also able to self-renew and replenish dividing cell populations within the layers when required, and are therefore termed stem cells.

Figure 1.1. ***Simple diagrammatical overview of the three germ layers and the specialized cells arising from their differentiation.***

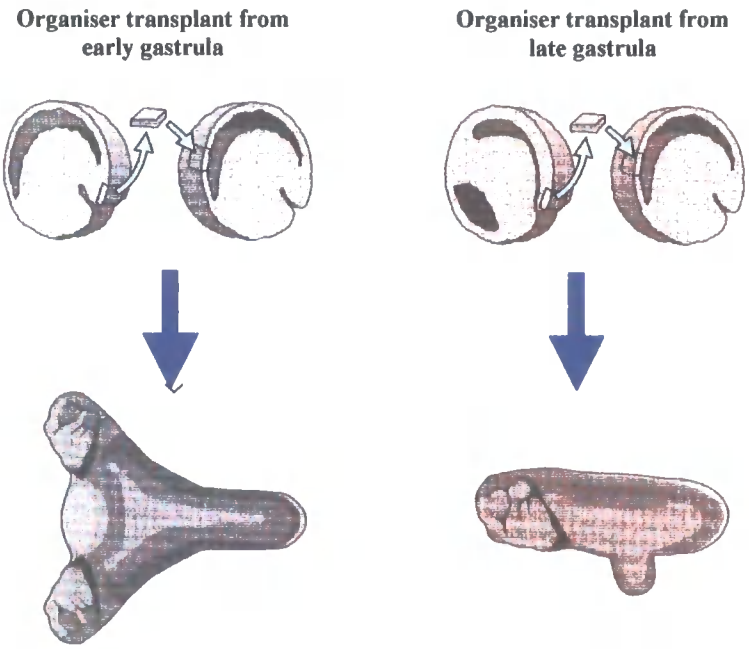


In the developing embryo cell fate determination is firstly modulated by various maternal factors. However, the final orientation of the body plan primarily occurs as a result of communications between cells or to a lesser extent by signal transmissions across the different germ layers. This is a critical process if the correct body axis is to be established. One area in which this cell fate determination is particularly well researched is in the ectoderm. In the vertebrate ectoderm, ventral stem cells differentiate into epidermis and the dorsal ectoderm differentiates into the components that make up the central nervous system (CNS). Spemann and Mangold first carried out studies on the amphibian, *Xenopus Laevis,* which led to their proposal that neural induction occurred in the ectoderm in 1924. In one study Spemann and Mangold grafted a region of the blastopore containing the dorsal lip onto the ventral side of a gastrula newt. This grafted area developed an entire second body axis (Figure 1.2a). Within this new body axis a secondary nervous system was established using cells from the host's ectoderm, which would normally have formed the epidermis (Figure 1.2b). Spemann and Mangold concluded that neural induction could be induced in the embryonic ectoderm and that the signals required for this induction were found in the dorsal lip (latterly termed the Spemann organizer).

Figure 1.2:

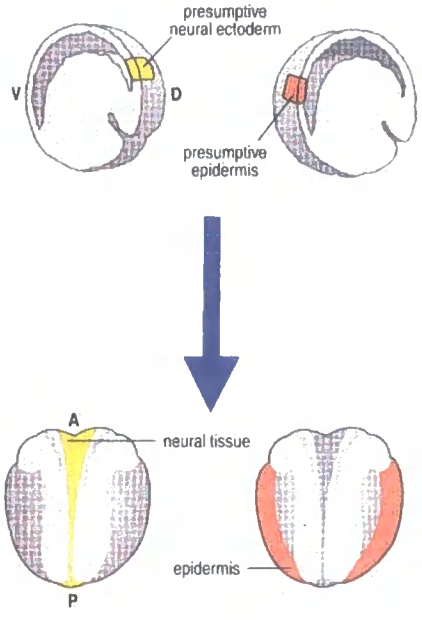
Diagrammatic overview of Spemann and Mangold's conclusions from their studies of ectodermal patterning in Xenopus laevis. *a) A graft was taken from the blastopore of a early gastrula frog, containing the dorsal lip, and transplanted onto the ventral side of a gastrula newt, resulting in the formation of an additional body axis (left panels). This does not occur in grafts of late gastrula material (right panels). b) Normal ectodermal development is shown in the left hand panels where dorsal ectoderm forms epidermis and ventral ectoderm forms neural tissue. If ectoderm from the dorsal area is transplanted into the ventral area during early gastrulation, the transplanted tissue forms neural cells. This shows that neural tissue is induced during gastrulation. (Diagrams modified from Principles of development; Wolpert L et al 1998)*

a)



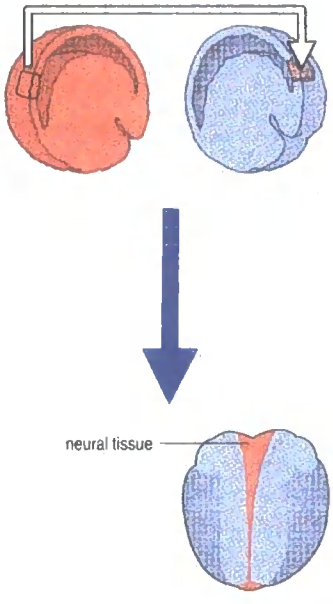
b)

Early gastrula



Normal fate of ectoderm at neurula stage

A piece of presumptive epidermis is transplanted onto another gastrula into the presumptive neural ectoderm



Induction of neural tissue in transplanted fragment.

1.1.2 Discovery of the Default Neural Pathway:

After Spemann and Mangold's original studies in 1924, further experiments identified several neural inducers that are expressed and secreted from the Spemann organizer. The most notable of these inducers are noggin, follistatin and chordin, all of which are able to induce neural tissue formation directly. The neural inducing properties of noggin were first discovered by Lamb et al. in 1993, they showed that noggin could induce anterior brain markers but not hindbrain or spinal cord markers which was the expected expression profile of an endogenous neural inducer as this is the area where neural formation takes place. Similarly follistatin was shown to be expressed in gastrula in the Spemann organizer and was able to induce neural tissue types in animal caps with the absence of any mesoderm (Hemmati-Brivanlou et al 1994). In further experiments Sasai et al 1994 demonstrated the neural inducing properties of chordin where they went on to show that both the injection of chordin mRNA and chordin as a soluble protein resulted in the direct formation of neural tissues.

Since the initial discovery of neural induction by Spemann and Mangold (1924) it was universally considered that stem cells in the ectoderm would differentiate in an epidermal direction unless instructed otherwise, i.e. epidermal differentiation was the default pathway. However Grunz and Tacke (1989) were one of three groups to challenge this view. They discovered that if cells in the early gastrula ectoderm of the *Xenopus laevis* were disaggregated and not exposed to any inducing factors, and kept in this state for up to 5 hours before being reaggregated, the cells would differentiate in a predominantly neural direction. This study suggested neural formation was the default pathway, and that in normal circumstances the ectoderm formed epidermis as a result of inhibitory factors suppressing this default neural differentiation pathway. This theory was supported by the work of

Hemmati-Brivanlou and Melton (1994), when they injected a truncated activin type II receptor into *Xenopus* animal caps, resulting in the production of neural tissues. This mutated ligand has been shown to block the activity of activin and other ligands associated with the transforming growth factor-β (TGF-β) super-family in a dominant inhibitory fashion. Hemmati-Brivanlou and Melton (1994) concluded that the mutated receptor bound to the normal ligand and rendered the complex inactive, suggesting that the formation of neural tissue could only occur if the organizer secretes factors that inhibit TGF-β signalling, otherwise epidermis is formed.

1.1.3 The Bone Morphogenetic Protein Signalling Pathway:

As the default pathway was now considered to be neural, scientists began the search to identify an inhibitor of this pathway, which presumably must be present throughout most of the ectoderm where formation of epidermis takes place. Wilson and Hemmati-Brivanlou first reported finding such an inhibitor in 1995. They hypothesized that the neuralisation of the animal caps in previous experiments was a result of diluting away the tonic epidermal inducer, therefore if the inducer was re-introduced, epidermal differentiation would result. In the light of the earlier work with the mutated activin receptor (Hemmati-Brivanlou and Melton 1994), Wilson and Hemmati-Brivanlou concluded that members of the TGF-β super-family were possibly good candidates for epidermal inducers. In cell dissociation assays, the ectoderm was dissociated and soluble factors, which were related to the TGF-β super-family, were added to the cultures in an attempt to induce the epidermal lineage. Activin was found to inhibit neuralisation, however, it did not induce epidermal differentiation, but induced mesoderm. Eventually it was discovered that bone morphogenetic proteins (BMPs), which are related to the TGF-β super-family, especially BMP4 and to a lesser extent BMP2 and 7, were strong epidermal inducers (Wilson and Hemmati-Brivanlou 1995, Suzuki et al

1997). In both reports, BMPs were found to be present in the embryonic ectoderm, appearing to inhibit neuralisation and also promoting the formation of epidermal derivatives. In support of these initial studies were experiments that blocked BMP activity in intact ectoderm resulting in the formation of neural tissues, suggesting that in normal circumstances, BMP signals inhibit neural induction (Hawley et al 1995). Figure 1.3 provides an overview of the findings of these investigations.

The discovery that BMPs were involved in the inhibition of neural cell fates and were considered to be inducers of epidermal lineages, led to the notion that neural inducers secreted *in vivo* by the Spemann organizer are likely to work by blocking BMP signalling. Studies in *Xenopus* on noggin and chordin, established that they were both able to bind directly with BMPs with a higher affinity than that of the normal ligand receptor complex (Zimmerman et al 1996, Piccolo et al 1996). This interaction prevents BMPs from binding to their receptors, inhibiting any signal transduction along the BMP signalling pathway that would result in the inhibition of the default neural pathway. At present it is uncertain whether follistatin disrupts the BMP signalling in a similar way, but it is known follistatin does interact with numerous BMPs, including BMP-4 (Fainsod et al 1997). Follistatin appears to form an inactive trimer between itself, the BMP, and the BMP receptor thereby blocking any signal transduction (Iemura 1998).

Down stream components of the BMP signalling pathway have also been shown to induce epidermal derivatives in dissociation assays (Massague 1997). Initially, BMPs bind cooperatively to type I and type II TGF-β receptors. Subsequent to activation of the BMP receptor, intracellular Smad proteins transmit the signal downstream (Massague 1997). Smad proteins constitute a large family of proteins that are able to transduce signals for different TGF-β ligands. Smad1 and Smad5 and possibly Smad9 appear to play a positive role in the activation and transmission of the signals along the BMP pathway. They have

been shown that when they are overexpressed they are able to induce epidermal derivatives in dissociation assays as well as inhibiting neural formation (Wilson et al 1997). Smad4 alone does not appear to be able to transduce signals for any TGF-β ligand, but it does seem to be a common partner essential for the signal-transducing Smads (Zhang et al 1997).

While Smad proteins 1, 5 and 9 appear to work in concert with the BMPs, and promote the formation of epidermal tissues, another group of Smad proteins (Smad6, 7, and 8) have been cloned which have been shown to inhibit the activity of the TGF-β ligands (Massague 1998). Smad6 seems to block the signalling pathway by binding to Smad1 and rendering it inactive, Smad7 disrupts the signalling by inhibiting all TGF-β ligands, including those used by the BMP signalling pathway (Hata et al 1998). Smad8 also blocks TGF-β ligands, but only those ones specific to the BMP signalling pathway. In all cases overexpression of these Smads is able to induce neural differentiation in experiments using *Xenopus* animal caps (Hayashi et al 1997). It appears that these anti-Smad proteins may be important in the regulation of the amount of BMP signalling in any one cell at any one time.

Active Smad1/Smad4 complexes are able to translocate into the nucleus of the cell and from there they activate the expression of various genes that encode for a number of transcription factors including *msx1, gata-1* and *vent.* These factors are able to inhibit neural differentiation and induce the expression of particular genes which result in the formation of the epidermis. The activity of molecules associated with the BMP signalling pathway is summarized in Figure 1.4.

Figure 1.3: ***Cell fate determination in the ectoderm of the Xenopus laevis embryo.*** *When the ectodermal explant (animal cap) of the blastopore was cultured in buffered saline it formed epidermis. However if the Spemann organizer was present in the culture the cell fate switched to neural tissue, thereby backing up claims that the organizer secretes neural-inducing factors, later identified as noggin, follistatin and chordin, which are responsible for CNS development in vertebrates. If the animal cap is dissociated and maintained in this state in Ca^{2+} and Mg^{2+} free buffer for up to 5 hours, the resulting cellular differentiation is in a neural direction. This indicates that with the presence of no outside inducing agents the animal cap will take on a neural call fate. In other words neural cell fate is the default pathway in the embryonic ectoderm. When dissociated cells were incubated with bone morphogenetic proteins (BMPs), epidermis was the resulting product after the cells had been reassociated, implying that BMPs inhibit default neural induction. This observation seemed to indicate that in the ectoderm, BMP signalling may be responsible for inhibiting neural differentiation and inducing epidermal development.*

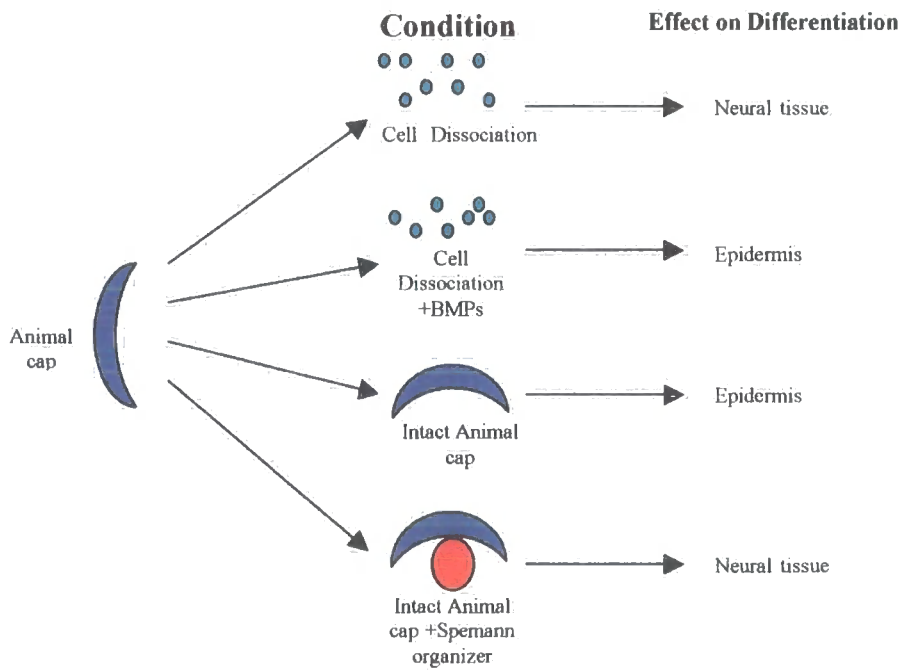
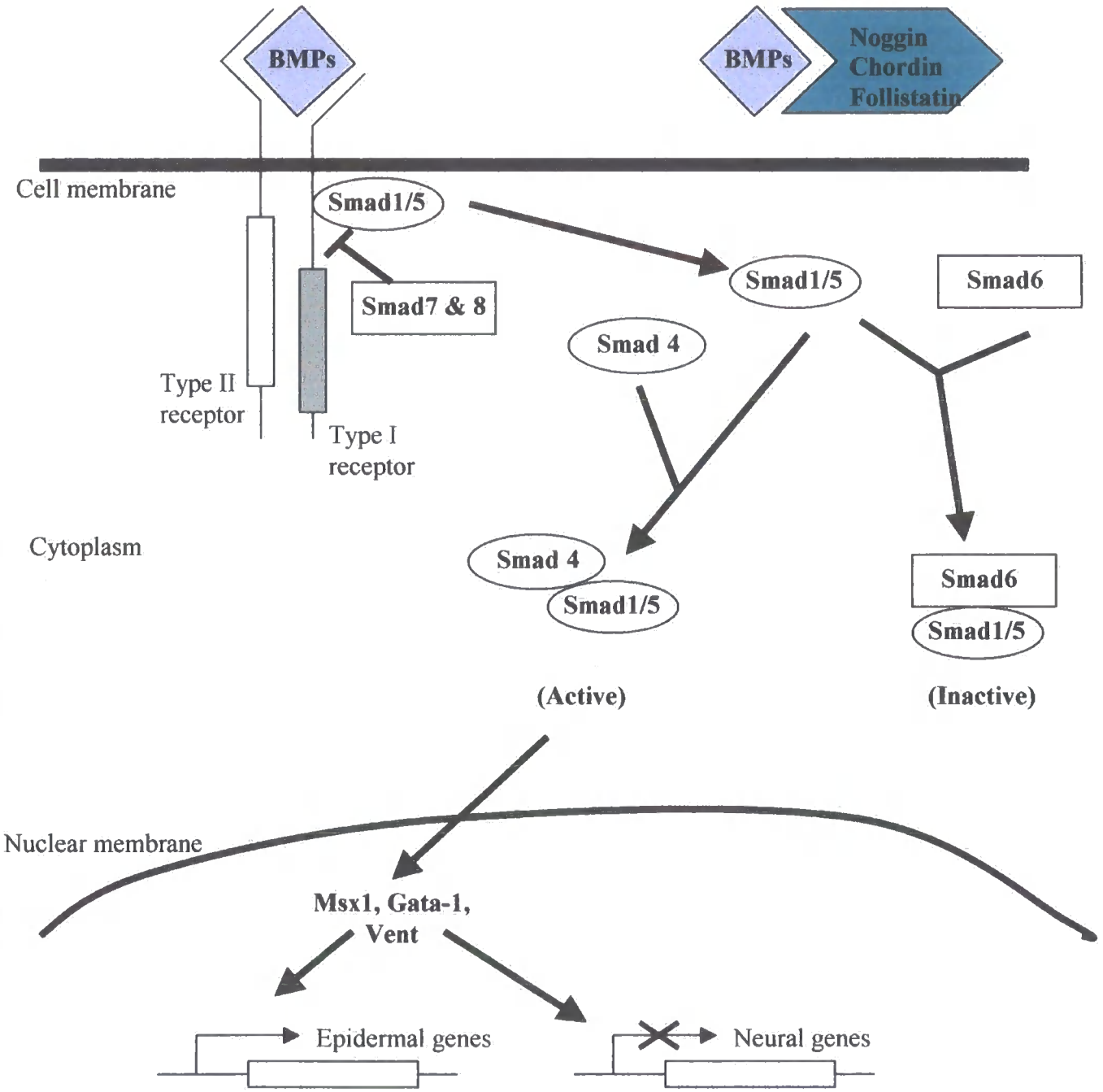


Figure 1.4: ***Overview of the BMP signalling pathway.*** *BMPs bind to type I and II TGF-β receptors on the cell surface membrane which activate intracellular Smad proteins. Activated Smad1 forms a complex with Smad4 which is then translocated into the nucleus. The complex activates specific transcription factors such as msx1, gata-1 and vent that in turn initiate the expression of epidermal genes leading to the formation of epidermis. This complex also represses the expression of neural genes, inhibiting neural formation. The pathway can be blocked at several stages. Neural inducers secreted from the Spemann organizer, such as noggin and chordin bind directly with BMPs stopping them from binding with their receptors. Within the cell, Smad7 and 8 prevent activation of Smad1, and Smad6 stops the formation of the Smad1/4 complex by binding to Smad1 rendering it inactive. All methods serve to modulate the amount of BMP signalling received by any one cell. (Smad proteins in the oval shapes aid the transmission of BMP signals, whilst Smads in the rectangles antagonize this signalling pathway).*



1.1.4 Conservation of the Default Neural Pathway:

Most of the studies reviewed so far were carried out in *Xenopus laevis*, however the conservation of the basic mechanism findings involved in cell fate determination in the ectoderm appears to be high across many species. For instance, studies in *Drosophilia* revealed that although neural lineages arise from the ventral ectoderm, BMP activity must still be inhibited for this process to take place (Ferguson and Anderson 1992). Homologs of BMP2/4 have been identified in *Drosophilia,* along with homologs for chordin and are termed *decapentaplegic (dpp)* and *short gastrulation (sog)* respectively. Inhibition of *dpp* results in the formation of neural cell types which would, under normal conditions, have formed the epidermis (Ferguson and Anderson 1992). It has also been shown that *sog* and *Xenopus noggin* antagonize *dpp* activity before it binds to the *dpp* receptor. This reinforces the hypothesis of the default neural pathway, and showing that it is conserved between species as diverse as *Drosophilia* and *Xenopus* (Holley et al 1996). At present there is a lack of data about inhibition of the BMP pathway and its relevance in neuralisation in higher vertebrates. However, chick, fish and mammal equivalents to the Spemann organizer have been identified that induce a secondary body axis when transplanted into separate blastocysts (Waddington and Schmidt 1933, Oppenheimer 1936). Furthermore, homologs to the neural inducers noggin, follistatin and chordin have also been found to be expressed by the ectoderm in higher vertebrates (Waddington and Schmidt 1933, Oppenheimer 1936), including humans (Beddington 1994).

Mutations in the zebrafish have uncovered similar findings to those seen in *Xenopus* studies. A null mutation exhibited in the BMP2 gene appears to result in enlarged neural plate formation (Kishimoto et al 1997), whereas a null mutation in *chordino*, a homolog of chordin, results in a dramatic reduction in size of the neuroectoderm (Schulte-Merker et al 1997). These data further support the theory that BMPs inhibit the formation of neuronal

tissues as well as inducing epidermal production and are therefore essential for the normal ectodermal development. Furthermore, these studies add more weight behind the argument that the neural default pathway exists and also show that this mechanism appears to be highly conserved between *Xenopus* and many other different species.

Experiments carried out in murine cell lines tried to establish whether the default neural pathway model was also conserved during mammalian embryonic development. At the time the model culture system of choice was the P19 pluripotent mouse embryonal carcinoma (EC) stem cell line. P19 EC cells can be induced to form neurons with the introduction of retinoic acid into the culture medium (Hoodless and Hemmati-Brivanlou 1997). When BMP4 was also introduced to cultures, retinoic acid induced neuronal development was inhibited and an increased expression of markers for epidermis was recorded (Hoodless and Hemmati-Brivanlou 1997). These data further indicate that neuronal development may occur due to the antagonism of the BMP signalling pathway. Supporting this argument was evidence that showed that transfection of the truncated activin receptor (similar to that used in the *Xenopus* studies) or of follistatin into undifferentiated P19 EC stem cells resulted in neural differentiation (Hoodless and Hemmati-Brivanlou 1997, Fainsod et al 1997). At present research has being carried out to try and establish successful knock-out mouse models which target deletions in the BMP signalling pathway. When a successful model has been created the hope is that it will be able to determine the extent of the role which BMPs play in the inhibition of neuronal development within the ectoderm in mammals. So far models have been created which have eliminations of either BMP2, BMP7, the BMP receptor or Smad4 (Zhang and Bradley 1996, Dudley et al 1995, Mishina et al 1995, Sirard et al 1998). However, none so far have been very informative due to either being lethal or having little effect on ectodermal development (reviewed in Weinstein and Hemmati-Brivanlou 1999).

Whilst there is compelling evidence suggesting that the mechanisms that function to determine cell fate in the ectoderm are conserved in vertebrates, there are significantly less data describing their activity in humans. Obviously, ethical and moral issues surrounding the use of human embryonic and foetal tissues limit the extent of research into this area. Moreover, there is a lack of suitable and readily available model systems to explore this mechanism in vitro. During this investigation, I describe the development of an in vitro cell-based system that has the potential to enable researchers to study the formation of neural and epidermal lineages in man. This study shows that a cell line created from embryonal carcinoma cells have the ability to differentiate into either a neuronal or epidermal lineage in response to certain factors.

1.2 Embryonal Carcinoma Stem Cells: A Model of Mammalian Embryogenesis

Embryonal carcinoma (EC) cells have long been understood to be the pluripotent stem cells whose differentiation can lead to the many differentiated cell types found in germ cell tumours (GCT). EC cells are most commonly found in tumours termed teratocacinomas, and they are understood to be the malignant counterpart of embryonic stem (ES) cells (Andrews *et al,* 2001). EC stem cells found in teratocarcinomas have been of great interest to pathologists for some time and have been shown to represent a researchable model of embryogenesis (Andrews *et al* 1987). The discovery that EC and ES cells have similar properties has helped overcome the many ethical and moral issues surrounding the study of human embryogenesis, which was greatly hampered due to the many ethical and moral issues surrounding the use of human embryonic material. EC cells are also a valuable medical tool that can be used to research a medically significant tumour type, namely testicular cancer.

1.2.1 Germ Cell Tumours

Teratocarcinomas are histologically complex tumours that belong to a class of neoplasms termed germ cell tumours (GCTs) (See Table 1.1). The most common form of this tumour type is the dermoid cyst of the ovaries. Such cysts develop from a totipotent germ cell, the primary oocyte, after it has been parthogenetically activated. In other words, the oocyte becomes self-activated and starts to develop as a fertilized zygote. During this process the oocyte starts to differentiate but after a while the cells become disorganized, forming a jumble of embryonic and foetal tissue types leading to the formation of a teratoma. Since teratomas arise from totipotent stem cells, they can give rise to many cell types necessary to form mature tissues in the body. Such differentiated tissues include clumps of hair, teeth, bone and sebaceous material (Figure 1.5). Dermoid cysts can grow very large in size, generally reaching about 10cm in diameter if not detected, but generally remain benign. In contrast, testicular GCTs, which come about due to transformations of the primitive germ cells prior to meiosis, are usually highly malignant.

Generally speaking testicular tumours can be split into two sub-groups, seminomas and non-seminomas GCT. A seminoma is described as a homogeneous GCT tumour containing uniform cells that resemble primordial germ cells. Non-seminoma GCT are described as heterogeneous tumours, they may contain one or more of the cell types that resemble extraembryonic tissues of the trophoblast or yolk sac, (i.e. choriocarcinoma or yolk sac carcinoma cells, respectively).

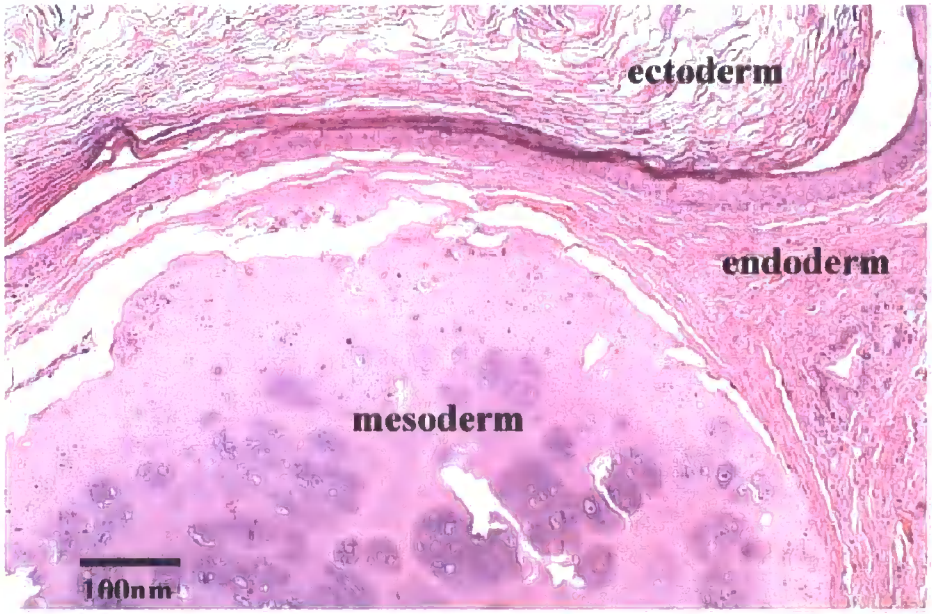
Table 1.1: Simplified description of the various components and features of Germ Cell Tumours. *(Derivatised from Andrews et al. 2001)*

Histological Type	Description	Comments
Teratoma	A tumour that contains an array of differentiated somatic cell types	The tumour may contain organised well-recognizable anatomical structures such as teeth. These tumours are often benign, but the malignant potential is well known. The most common form is the benign dermoid cyst of the ovary
Embryonal carcinoma	'Undifferentiated' epithelial cells resembling embryonic cells of the ICM and the primitive ectoderm.	Highly malignant tumours, the cells are generally regarded as stem cells able to differentiate into a range of histological cell types.
Teratocarcinoma	A tumour that contains both teratoma and embryonal carcinoma.	The malignancy of these tumours is due to their embryonal carcinoma component.
Yolk sac carcinoma	Cells resemble those of the extraembryonic 'yolk sac'	In mouse tumours cells may resemble parietal or visceral yolk sac, in humans no such clear distinction is evident.
Choriocarcinoma	Cells resemble cyto- and syncytiotrophoblast of the placenta.	These do not occur in GCTs of the laboratory mouse.
Seminoma	Relatively uniform cells resembling 'primordial germ cells'	A malignant tumour that does not occur in the laboratory mouse. Known as Dysgerminoma in females.

Figure 1.5*: Structural features of a teratoma: A: Low power microscopic appearance of teratoma. The three embryologic germ layers are represented by skin (ectoderm), cartilage (mesoderm), and a colonic gland (endoderm) (www.medlib.med.utah.edu/WebPath/PEDHTML/PED050.html).*

B: Gross appearance of a benign ovarian dermoid cyst. www.kumc.edu/instruction/medicine/pathology/ed/ch_18/c18_dermoid_gross.html.

A:



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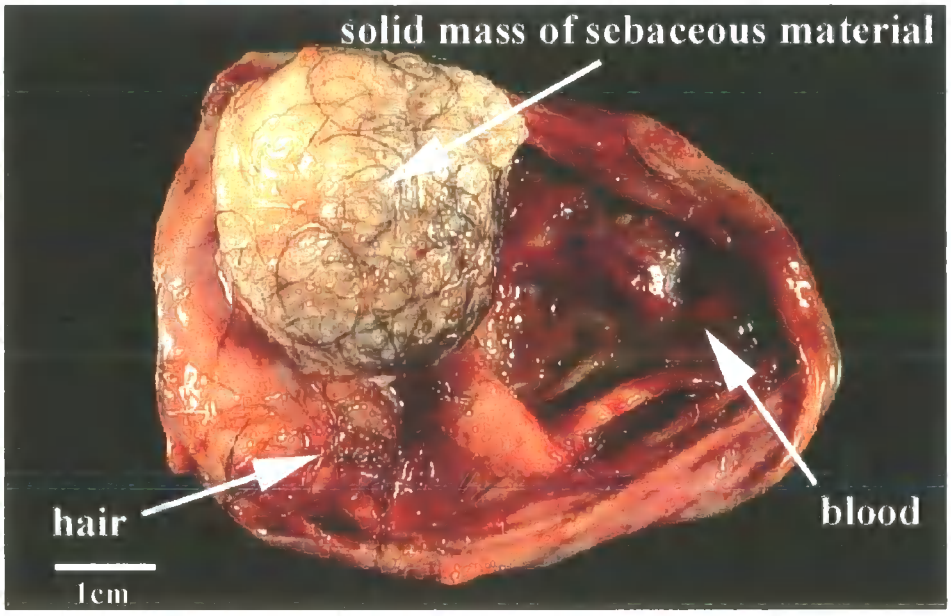
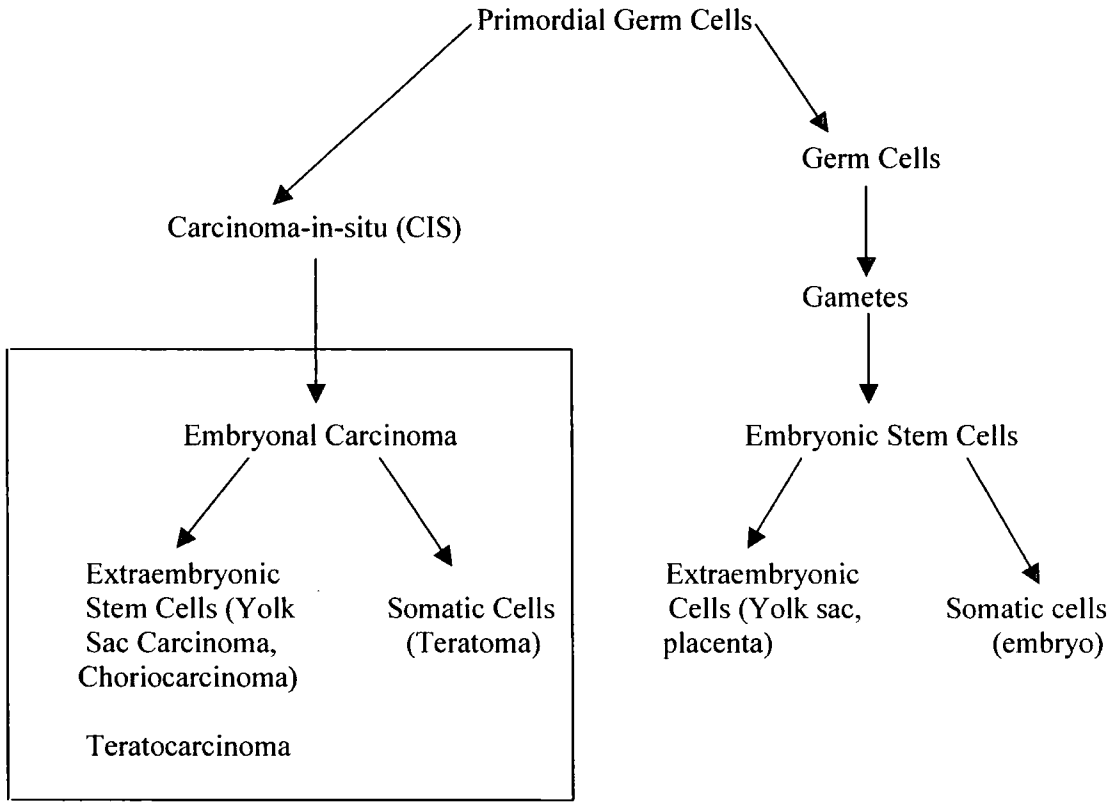


GCT can also contain other tissue types such as nerve, bone or muscle similar to their teratoma counterparts. Most testicular GCTs also contain undifferentiated epithelial cells which resemble the embryonic cells of the inner cell mass (ICM) and primitive ectoderm. These undifferentiated epithelial cells have been deduced by studies reporting on their biochemical and immunological properties, as well as in their developmental potential (Andrews *et al* 1983). The undifferentiated epithelial cells are the EC cells, and it is these cells which give the tumours there malignant characteristic. Kleinsmith and Pierce (1964) conducted experiments proving the malignancy of such EC cells. They transplanted a single murine EC cell into a suitable mouse host and discovered that a whole new teratoma or teratocarcinoma formed. These studies also showed the ability of EC cells to differentiate into the wide range of tissue types found in the tumour. In most cases the differentiated products of the EC cells have a limited proliferative ability and have lost their malignant properties.

The term teratocarcinoma is generally given to GCT that contains both embryonic tissues (teratoma) and EC cells. Teratocarcinomas of the testis are the main tumour type used by developmental biologists for research purposes due to their apparent similarity to embryogenesis and the fact they arise from germ cells. Figure 1.6 summarizes the close relationship of GCTs to their normal germ cell and embryonic counterparts.

Figure 1.6: ***Diagram depicting the similarities between normal germ cell and their malignant counterparts, germ cell tumours.*** *In normal embryogenesis the primordial germ cells follow a specific pathway creating embryonic stem cells which then differentiate into the extraembryonic and somatic cells. The tumour production follows a similar pathway resulting ultimately with corresponding differentiated cells. (Modified from Andrews et al, 2001)*



1.2.2 Historical Studies of Embryonal Carcinoma Cells

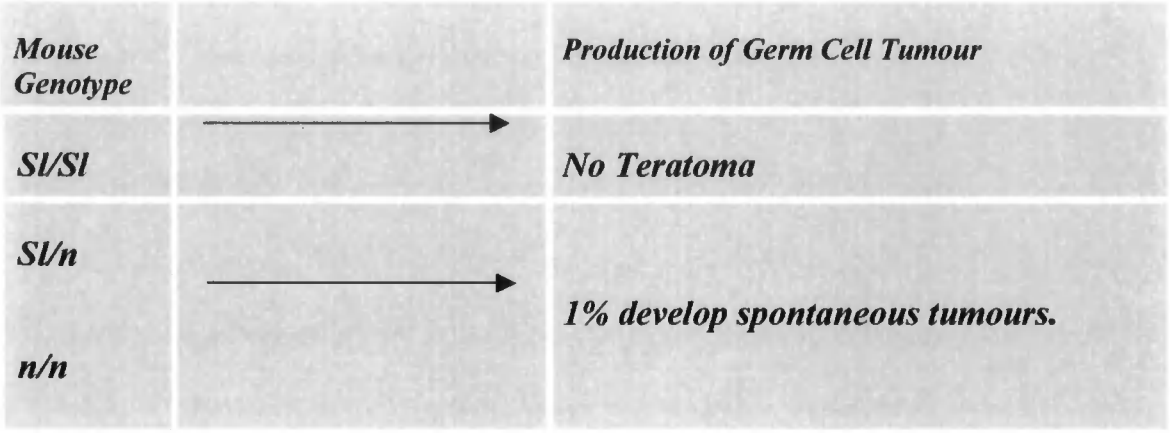
The importance of EC cells becomes apparent when discoveries made by Solter and Damjanov (1979), and Andrews (1983), using EC cells, led to ground-breaking research in isolating murine embryonic stem (ES) cells (Evans and Kaufman, 1981; Martin, 1981), then ultimately to the isolation of human ES cells (Thomson, 1998).

It is suggested that the formation of teratocarcinomas reflects the processes of embryonic development. It was therefore thought that EC cells might resemble undifferentiated stem cells that are found in the early embryo. However the study of this concept was hampered due to the rarity of such tumours occurring. This problem was in part overcome with the discovery that provided a model system by Stevens and Little (1954). They reported that 1% of male mice of strain 129 develop testicular teratomas. Stevens (1964) also discovered that the incidence of the tumours occurring could be increased by transplanting the genital ridges of early embryos to the testis capsule of adult mice. There is a only a short time period in development when the embryonic genital ridges are susceptible to such transplantation, and in the mouse this is from 12 to 12.5 day old embryos. Solter and Damjanov (1979) also reported that these testicular GCTs could be induced in other strains of mice from ectopically transplanted embryos.

The testicular GCTs forming either spontaneously in 129 mice or following transplantation methods can be split into two groups. One group contains the tumours that can be maintained as undifferentiated EC cells *in-vitro*, but are still able to form teratocarcinomas when transplanted back into syngeneic mice hosts. These cells are said to be pluripotent. The other group contains the tumours which are non-retransplantable. These are known as teratoma tumours and do not contain any EC cells (Andrews *et al* 2001).

Stevens (1967) studied mice homozygous for mutations at the *steel (Sl)* locus to prove that teratocarcinomas were derived from germ cells. *Steel* encodes for stem cell factor, which is a growth factor essential for the survival of the primordial germ cells when they migrate through the genital ridge of the developing embryo. Mice homozygous for the *Sl* gene have no stem cell factor and are therefore infertile due to the fact that the primordial germ cells do not survive during migration. In this study, Steven's crossed the *Sl* locus on to the 129 mouse background. The *Sl* experiments concluded that all of the genital ridges of *Sl*/*Sl* mice on a 129 background did not produce any teratomas when, as investigated and proved before by Stevens and Little (1954), 1% should have produced tumours (Figure 1.7). This data therefore supported the hypothesis that the origin of the teratoma and teratocarcinoma is likely to be the primordial germ cell (Stevens, 1967).

Figure 1.7:



Evidence produced by Stevens (1967) concluded that the primordial germ cell is the origin of teratomas and teratocarcinomas: Mice homozygous for the steel (Sl) gene (Sl/Sl) are found not to produce teratomas of the testis, where, as it has been proved before, 1% should develop spontaneous tumours. This 1% tumour development is seen with mice heterozygous for the Sl (Sl/n) gene or who have a normal (n/n) genotype. The Sl gene encodes for stem cell factor, which is essential for primordial germ cell survival as they migrate through the genital ridge of the developing embryo. Mice homozygous for the Sl gene produce no stem cell factor thus no primordial germ cells would survive the migration, and given the complete absence of a GCT, it can be concluded that the origin of a teratoma or teratocarcinoma is most likely to be the primordial germ cell.

1.2.3 Embryonal Carcinoma in the Laboratory Mouse

- **Murine Embryonal Carcinoma Cell Lines**

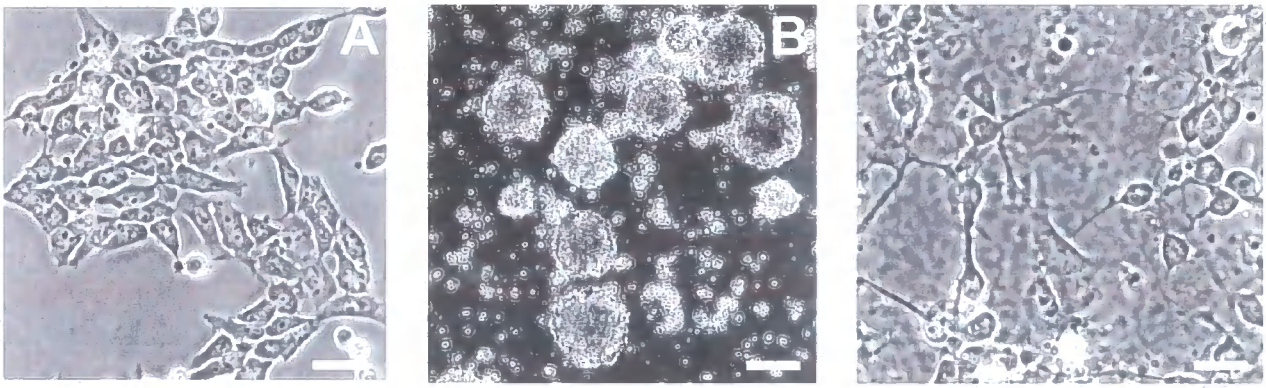
The increasing importance of EC cells in both the study of cancer research and embryogenesis lead to the derivation of several cell lines for research purposes. In essence, EC cells were isolated from murine teratocarcinomas then seeded onto plates and cultured. This type of work was originally pioneered by Kahn and Ephrussi (1970). Since then many others, such as Pfeiffer *et al* (1981) and Jones-Villeneuve *et al* (1982), have also established murine EC cell lines. A typical feature of many of the established lines is that they can be maintained as undifferentiated EC cells *in-vitro,* but when they are transplanted back into a suitable mouse host they form the many differentiated tissue types that are associated with teratomas. Cultured EC cells are therefore still capable of differentiation into multiple tissue types. On this basis, such cell lines are said to contain 'pluripotent' EC cells. However, some EC cell lines seem to lose the ability to differentiate and are therefore termed 'nullipotent'. This is understood to be due to mutations within the EC cells themselves. Furthermore, such genetic mutations are likely to give EC cells their malignant properties, but it may also interfere with the cells ability to differentiate, i.e. they are selectively at an advantage if they can mass replicate themselves to produce more tumour cells.

▪ Differentiation of Murine Embryonal Carcinoma Cells

Subsequent to the establishment of murine EC cell lines, scientists actively investigated their propensity for differentiation. Investigation into the mechanisms of cell differentiation would have many useful implications for developmental and cancer biologists, alike.

Some pluripotent EC cell lines could spontaneously differentiate in culture. For example, if the murine cell line PCC3 was allowed to reach confluence in culture and was maintained in that state for several days then the cells started to differentiate into a variety of cell types including nerve and muscle (Andrews *et al,* 2001). Indeed, many of the early studies of EC cell differentiation were carried out with spontaneously differentiating cell lines, mostly by the differentiation of embryoid bodies that were produced when the cells were cultured in suspension (Martin and Evans 1975). However, the study of this uncontrolled differentiation made it difficult to analyse the underlying biochemical processes and mechanisms involved. Therefore, a breakthrough for this problem came with the discovery that the apparently nullipotent cell line F9 could be induced to differentiate when the cultures were exposed to retinoic acid (RA). After exposure to RA the cells differentiated into cells that resembled parietal endoderm (Strickland *et al* 1980). This discovery highlighted the importance of retinoic acid for the processes of regulating embryonic development. Other cell lines were also induced to differentiate when exposed to retinoic acid, these lines included PCC7 (Figure 1.8) (Pfeiffer *et al* 1981), and also P19, one of the most studied line in this area (Jones-Villeneuve *et al* 1982). The P19 cell line was found to differentiate into neural cell types when exposed to retinoic acid and muscle cell types when exposed to dimethylsuloxide (DMSO). It could also be induced to differentiate into other cell types when exposed to alternative substances such as hexamethylene bisacetamide (HMBA) (Andrews *et al,* 1990).

Figure 1.8:: ***Mouse PCC7 embryonal carcinoma stem cells and their differentiated derivatives:*** *A: Undifferentiated PCC7 pluripotent embryonal carcinoma cells B: Embryoid bodies formed by culture in suspension. C: Differentiation into neurons and other cell types after induction with retinoic acid. Bars A: 20μm B:100μm C:15μm (Courtesy of Dr S Przyborski.)*



- **Relationship of Murine Embryonal Carcinoma Cells to the Early Embryo**

With the establishment of murine EC cell lines during the sixties and early seventies the hypothesis that EC cells resemble the stem cells found in the early embryo was tested. At the time, one of the many methods used involved the relatively new technique of producing antisera to specific cell surface antigens. Boyse and Old (1969), claimed that the specific cell-surface antigens could play a vital role in the regulation of cell behaviour and differentiation. These ideas were quickly put into practice to identify specific embryonic cell-surface antigens with the intention to test whether EC cells expressed the same cell-surface antigens as their supposed embryonic stem cell counterparts, therefore providing further evidence in support of this hypothesis.

Artzt *et al* (1973) was one of the first groups to put the use of antisera into practise using EC cells. The method they used involved immunising adult 129 mice with EC cells of the F9 line that were also of 129 origin. As the implanted EC cells were 'foreign' the host elicited an immune response producing antibodies specific to the embryonic EC cells that could be collected and studied. When tested by cytotoxicity and immunofluorescence assays, the anti-F9 sera produced from immunized 129 mice was found to express antigens found on EC cells but that were not expressed on a range of other differentiated cells. The same antigens were also discovered to be expressed by cells of the inner cell mass (ICM) of the embryonic blastocyst, thus producing significant evidence that EC cells are the malignant counterpart of embryonic cells.

Subsequent studies backed up these initial findings with experiments using monoclonal antibodies. The initial findings using the anti-F9 sera were clouded with controversy at the time. This was due to the fact that anti-F9 sera was a polyclonal antisera and some groups

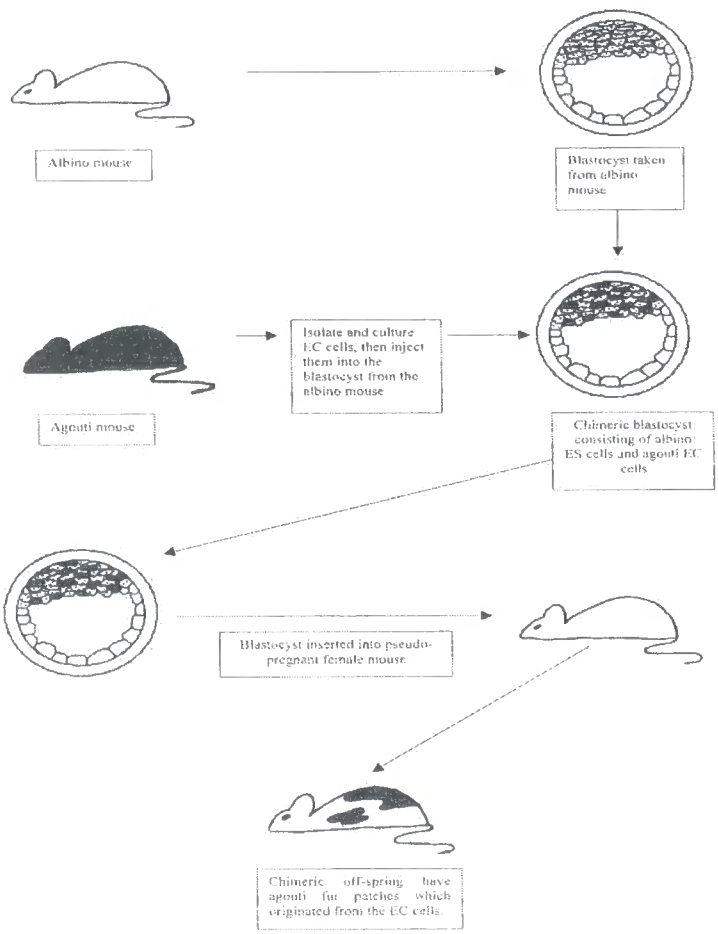
were questioning whether it was related to key cell-surface molecules encoded by the *T-locus* (Kemler *et al* 1976). One of the many difficulties of working with polyclonal antisera meant that the precise nature of this anti-F9 activity was never resolved, but a link to the *T-locus* became more and more unlikely as research advanced. However, the findings using the F9-antisera were strong enough to inspire further study in the area, and using anti-F9 sera as a starting point many reagents using monoclonal antibodies were produced that identified antigens with similar characteristics to the F9 antigen. The most interesting of these was the stage-specific embryonic antigen-1 (SSEA-1). This antigen was highly expressed both by murine EC cells and embryonic ICM cells. Moreover, it was likely that the polyclonal anti-F9 sera contained antibodies that recognised this epitope.

More direct evidence linking EC cells and embryonic stem cells came about when a small number of EC cells were transplanted into blastocysts and the grafted EC cells contributed to the formation of mature tissues found in the chimeric off-spring (Brinster 1974). In this study, a small number of EC cells were taken from an agouti mouse, these were then transferred into a blastocyst taken from an albino strain of mouse by micro-manipulation. The blastocyst was then implanted into a pseudopregnant female and some of the subsequent off-spring had patches of agouti fur indicating that the EC cells had become integrated with the stem cells in the embryo and contributed to the formation of the developing embryo, (Figure 1.9). Papaioannou *et al* (1975) extended findings and used single cell clones to prove that pluripotent EC cells can also participate in normal embryogenesis. Papaioannou's group injected the EC cells into the blastocoel cavity of a developing embryo, and were able to show that the pluripotent EC cells injected had differentiated into many of the tissue types within the chimeric mouse that developed. Mintz and Illmensee, (1975) provided additional evidence that suggested the EC component within a chimeric mouse was capable of extending to the germ line, bringing the relationship of EC cells to ES cells closer still.

Many of these studies involving murine EC cells have greatly increased the basic knowledge of the molecules and mechanisms that are involved with embryonic development and differentiation. Accordingly, EC cells have proven to be a robust and simple tool for the study of developmental biology.

Figure 1.9:

Experiments carried out by Brinster (1974) further backed up evidence linking EC cells to embryonic stem cells: *A small number of EC cells were taken from an agouti strain of mouse and were transferred into a blastocyst taken from an albino strain of mouse. The blastocyst was then implanted into a pseudopregnant female. The EC cells integrated with the stem cells in the developing blastocyst, resulting in some of the subsequent off-spring having patches of agouti fur. This experiment provided more direct evidence linking EC cells to embryonic stem cells.*



1.2.3 Embryonal Carcinoma in the Human

- **Human Embryonal Carcinoma Cells**

It was known that while there is strong conservation between mammalian embryogenesis including mice and humans, there still remain significant differences between different species. Accurate models of human embryogenesis will allow exploration of these differences in embryonic development of humans from other laboratory mammals.

Human EC cells provide a useful model system for this type of research, as while they resemble embryogenesis, they overcome many of the moral and ethical issues surrounding the use of embryonic tissues and ES cells. They are also an invaluable tool used in the study of a medically significant form of cancer.

One of the first human EC cell populations was isolated from a lung metastis originating from a testicular germ cell tumour (reviewed in Martin, 1980). The EC cells from this tumour were maintained as xenografts in mice, (Pierce *et al* 1957). Later, tumour tissues were explanted and grown in culture and EC cells were subsequently isolated and cultured directly *in-vitro.* One of the most well-known human EC lines created was given the name TERA2 (Fogh and Trempe, 1975).

Initially, studies reported the conserved properties between mouse and human EC cells. These early results showed that some of the human EC cell lines also expressed the F9 antigen. However, importantly, later comparative studies turned up interesting differences between the two cell types. It was detected that human EC cells did not express the SSEA-1 antigen, the hallmark of murine EC cells (Andrews *et al* 1982). Instead they were shown to express two new antigens, namely SSEA-3 (stage-specific embryonic antigen-3) and SSEA-4 (stage-specific embryonic antigen-4). These antigens had been previously been shown to only be expressed by cleavage-stage murine embryos, not by murine ICM cells or murine

EC cells. Other antigens have also been found to be expressed by the human EC cell surface, such as TRA-1-60, TRA-1-81 and GCTM2. TRA-1-60 has recently become a useful clinical serum marker in germ cell tumour patients, due to the fact that it is expressed by human EC cells, (Mason *et al* 1991).

- **Differentiation of Human Embryonal Carcinoma Cells**

One prominent feature in the early studies of the many human GCT-derived EC cell lines was their distinct lack of ability of many of the lines to differentiate into any well-recognizable cell types (Andews and Damjanov 1994). This could have been due to the fact that their ability to differentiate would limit the tumour growth so therefore putting those cells at a selective disadvantage to more malignant mutant types. This apparent inability of the human EC cells to differentiate made it difficult to define them as EC cells, due to the ability to differentiate being a key diagnostic feature of an EC phenotype. However this was soon overcome with the establishment of human EC cell lines that did undergo limited differentiation and that showed distinct morphological changes as well as changes in expression of various markers when the cells were exposed to different culture conditions. The early studies of human EC cell differentiation involved examination in closest detail of the 2102Ep human EC cell line (Andrews et al 1982). 2102Ep EC cells spontaneously differentiate when they were cultured at low densities, yielding a morphologically and antigenically distinct cell type. The differentiation of 2102Ep human EC cells at low cell densities results in the down-regulation of the surface antigen SSEA-3, and the appearance of SSEA-1. Differentiated 2102Ep cells have been found to resemble trophoblastic giant cells. Differentiation induced in cultures seeded at low densities could indicate that cell:cell contact is an important factor for maintaining the undifferentiated, malignant state. This is

backed up by the fact that the cell lines that show this low-density differentiation *in-vitro* do not appear to differentiate when grown as xenograft tumours (Andrews 1998).

The apparent trophoblastic differentiation of the 2102Ep cell line along with other human EC cells lines indicates a notable difference between murine and human GCT. This difference being the frequent occurrence of trophoblastic elements in human but not in mouse teratocarcinomas (Damjanov and Solter 1974). This finding fits with the theory that murine EC cells are the malignant counterpart to the late ICM, or primitive ectoderm cells which have lost the capacity for trophoblastic differentiation, explaining why it is rare to find trophoblastic elements in murine teratocarcinomas. Human EC cells are thought to correspond to an earlier stage of embryonic development than mouse EC cells, for example, to cleavage-stage embryos, therefore retaining the capacity for trophoblastic differentiation.

Along with the study of spontaneous differentiation in human EC cell lines many groups have investigated the ability of certain cell lines to differentiate under controlled culture conditions. For example, the initiation of differentiation by exposure to retinoic acid. The majority of pluripotent human EC cell lines share many of the typical features associated with EC cells in general (Andrews 1998), therefore strengthening the argument that, as in the mouse, human teratocarcinomas contain pluripotent stem cells that resemble those from the early embryo. The most extensively studied cell line in this area is the human pluripotent EC cell line known as TERA2.

- **TERA2 Human Pluripotent Embryonal Carcinoma Stem Cells**

The TERA2 EC cell line is one of the oldest and most extensively studied human GCT lines. It was derived in 1975 by Fogh and Trempe. Initially the TERA2 cell line was thought to be nullipotent. It took a few years to discover the pluripotent nature of the TERA2 cell line. This was due to the fact that TERA2 cultures are impure and frequently contain only a

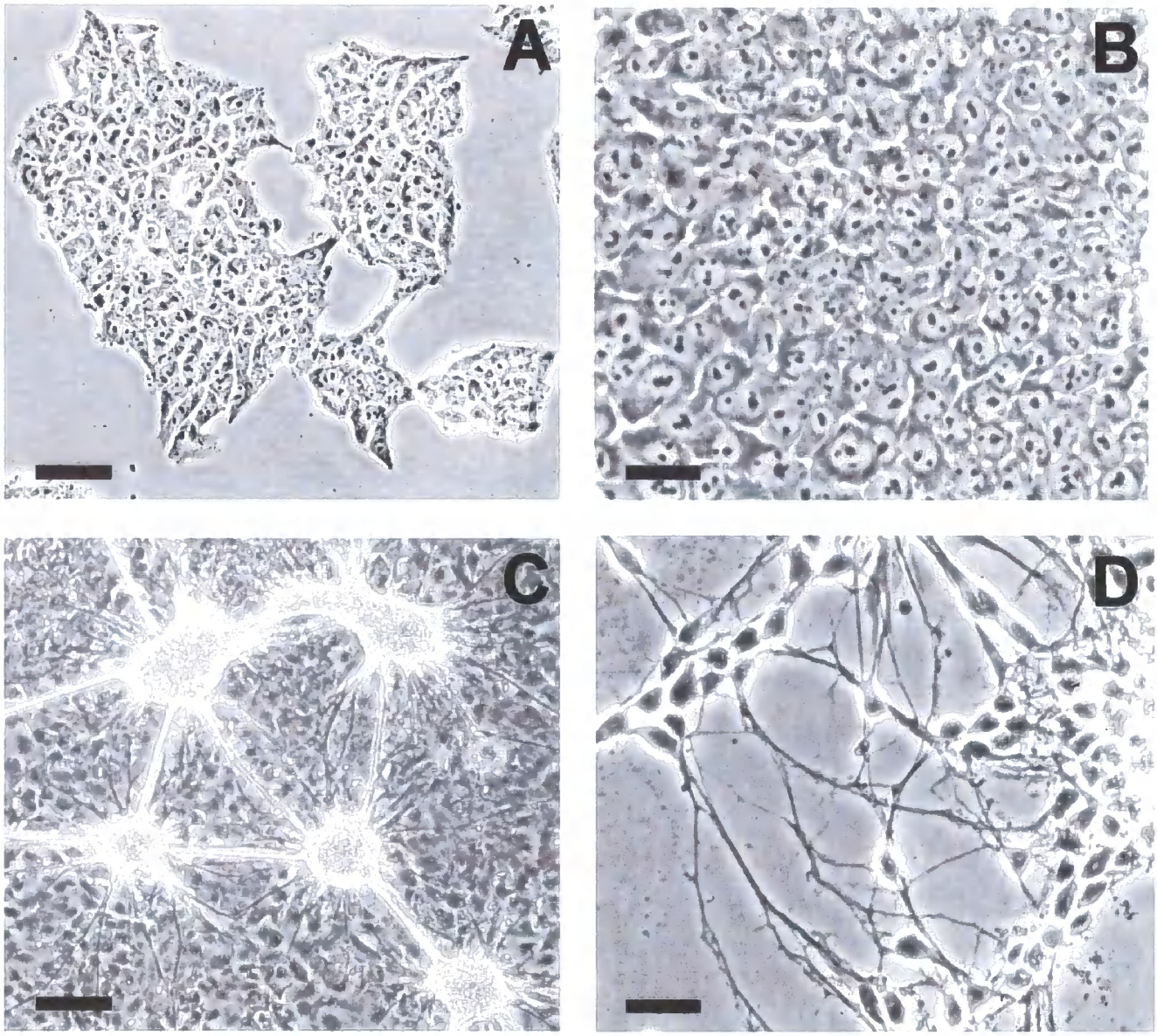
few EC cells. Obviously this impurity and lack of EC cells made it difficult to carry out any analysis on the EC cell component. A more robust, pure line was established by Andrews et al (1984) and was termed NTERA2. Andrew's group (1984) took TERA2 cultures and passaged them through an athymic *(nu/nu)* (nude) mouse. This resulted in the formation of a xenograft tumour containing glandular structures, mesenchyme and neural elements, as well as areas of EC cells. Both the TERA2 and NTERA2 cell lines have been shown to form well differentiated teratomas when transplanted into suitable hosts (Andrews 1984). Clones isolated from both TERA2 and NTERA2 cell lines have been shown to respond to retinoic acid and other agents in culture yielding both neurons and other non-neural cell types (Andrews 1984). However, the aim to create a pure EC cell line was not entirely reached. The NTERA2 cell line also contained contaminants, such as multiple cell types, most frequently being the glandular structures and neural elements found along-side the EC cells in the tumour. To combat this contamination problem single cell clones of NTERA2 were isolated, forming the widely used NTERA2 clone D1 (often abbreviated to NT2/D1). This clone was made up purely of EC cells with no contaminants. Cells were extracted from a xenograft tumour containing NTERA2 EC cells, then single cells were isolated using a micropipette from a suspension of trypsinized cells (single cell selection). The single cell was then transferred into a well of a 24 well plate. Small colonies were then derived from this well then cultured up slowly to form the NTERA2 clone D1 cell line (Andrews et al 1984).

The NTERA2 cell line and it's subsequent sub-lines express a range of characteristic surface antigens associated with other human EC cell lines such as 2102Ep, these include the antigens SSEA-3, SSEA-4, and TRA-1-60, a keratan-sulphate-associated glycoprotein antigen. NTERA2 EC cells also respond well when exposed to different agents in their culture media and induce differentiation. For example, NTERA2 EC cells commit to differentiation after only 1-2 days of exposure to retinoic acid. This was deduced since after

exposure to retinoic acid the cells rapidly lost their expression of the characteristic EC cell markers SSEA-3 and SSEA-4, and gained the expression of other cell surface antigens associated with the neural lineage, such as A2B5, a ganglioseries antigen (Andrews et al 1990). NTERA2 EC cells can also be induced to differentiate into distinct non-neuronal cell types by HMBA and by bone morphogenetic protein-7 (BMP7) (Andrews *et al* 1994). These findings therefore overcame arguments that NTERA2 EC cells are restricted to neuronal differentiation, however it is known that neuronal differentiation in this particular cell line is marked and the cell line can therefore be used as a valuable experimental tool for the study of neurogenesis (Przyborski et al 2000) (Figure 1.10).

Figure 1.10:

Human EC cells and their differentiated derivatives: *A: 2102Ep EC cells B: NTERA2 EC cells C: NTERA2 cells after exposure to retinoic acid, have formed neurons. D: Cultures of purified neural cells. Scale bars A,B,D: 25μm C:330μm. (Courtesy of Dr S Przyborski)*



- **NTERA2 Differentiation: The Effect of Retinoic Acid and Hexamethylene Bisacetamide**

Retinoic acid:

The study of EC cell differentiation has uncovered many details concerning the regulation and mechanisms involved in cell differentiation during mammalian development. As previously mentioned EC cells can be induced to differentiate with the introduction of certain chemicals into the growth media. Retinoic acid is one of the most effective chemicals to induce TERA2 EC cells and its sub-lines to differentiate. Retinoic acid is a derivative of vitamin A and is known to induce differentiation in many different cell types such as epithelial and cancer cells. It is also known to play an important role in the development of the central nervous system in the mammalian embryo, which may partly explain why it induces TERA2 EC cells to differentiate into neural-type cells. The effects seen after exposure of EC cells to retinoic acid are thought to be mediated by the interactions of cell ligand-receptor complexes with specific DNA response elements, such as the retinoid X response elements (Leid et al 1992). It is thought that such interactions result in the production of signalling pathways inside the cell causing them to alter the normal pattern of gene expression, and ultimately inducing the cell to undergo differentiation. EC cells express *hox* genes in response to retinoic acid induced differentiation. The most predominant being the 4.2 kB transcript of *Hox 5.1*, which otherwise is almost exclusively found in the central nervous system of the developing human embryo, therefore supporting the notion that retinoic acid induces neural derivatives from EC stem cells (Simeone *et al* 1990).

Using the human TERA2 cell line Andrews' group studied the glycolipids of the EC cells and their changes during chemically induced differentiation using retinoic acid as the inducing agent (Andrews *et al* 1990). This was achieved using monoclonal antibodies

specific to certain cell surface antigens. The study revealed that during and after differentiation induced by retinoic acid the expression of cell surface glycolipids undergo marked changes. The glycolipid antigens SSEA-3, SSEA-4 and TRA-1-60 (high molecular weight glycoproteins), which are expressed almost exclusively by pluripotent stem cells, are replaced on differentiated cells by lactoseries antigens such as SSEA-1, and ganglioseries antigens such as GT and 9-0-acetyl GD, which are recognised by the monoclonal antibodies A2B5 and Me311 respectively. Differentiated NTERA2 derivatives also express the monoclonal antibody VIN-IS-56, which is thought to recognize NCAM.

Further analysis of the differentiated cells shows that the different subsets of the cells can express different patterns of these antigens. For example, EC cells which have been exposed to retinoic acid over a long period of time (> 3 weeks), tend to form mature neurons. These neurons express the A2B5 antigen but not the ME311 or SSEA-1 antigen, indicating that A2B5 could be used as a neural marker of EC cell differentiation (Andrews *et al* 1990, Przyborski et al 2000). Indeed, A2B5 has been linked with neuronal cells (Eisenbarth et al 1979) however; there is no evidence of glial formation in the NTERA2 lineage (Andrews *et al* 1984).

Neurons derived from NTERA2 EC cells have been extensively studied. They have been shown to express all three of the neurofilament proteins, which are associated with the N-methyl-D-aspartate (NMDA) and non-NMDA glutamate receptor channels found in functioning neurons (Lee and Andrews 1986). The neural cells have also been shown to express muscarinic acetylcholine receptors and they are reported to be sensitive to glutamate excitotoxicity (Andrews 1998). Functional analysis has also concluded that the neurons produced are indeed functioning neurons capable of conducting electrical impulses (Rendt *et al* 1989). The neurons formed have been shown to bare closer relationship to embryonic neurons from the central, rather than peripheral nervous systems (Andrews 1998). The neurons created from the differentiation of NTERA2 EC cells have been reported to respond

to specific environmental cues to develop into distinct different types of neuron. For example, when the differentiated neurons were transplanted into different areas of a brain in immunodeficient mice the NTERA2-differentiated neurons took on the similar cell characteristics associated with the site of implantation (Kleppner *et al* 1995).

Other Inducers of EC stem cell differentiation:

Although the predominant differentiation pathway of NTERA-2 cell lines appears to be neuronal, the human EC cells can also be induced to follow other differentiation pathways when exposed to different inducing agents such as 5-bromouracil 2'deoxyribose (BUdR), 5-iodouracil 2'deoxyribose (IUdR), dimethylsulfoxide (DMSO) and hexamethylene bisacetamide (HMBA) (see below). The differentiation with each of the inducing agents was accompanied by the loss of the EC cell markers SSEA-3 and SSEA-4, however the antigen profiles seen after differentiation was significantly different from the profile obtained from retinoic acid-induced differentiated cells (Andrews *et al* 1986). For example, the antigen A2B5 appears in 60%-80% of NTERA2 cells differentiated with retinoic acid, but was detected in less than 20% of the cells induced by the other agents, therefore suggesting that the differentiated cells produced are different than the ones produced after exposure to retinoic acid (Andrews et al 1986). Andrews also showed that this has been revealed in EC cells induced to differentiate with DMSO, they appear to form muscle-like cell types as apposed to neural cell types seen after exposure to retinoic-acid.

Hexamethylene Bisacetamide:

HMBA-induced differentiation of NTERA-2 EC cells creates fully differentiated cells that appear similar to those non-neural cells induced by retinoic acid differentiation. They form homogeneous flat regular colonies when cultured in tissue culture plastic (Andrews *et al* 1986). It is not known whether HMBA induces the same form of differentiation as retinoic

acid. As mentioned, changes in cell surface antigen expression and glycolipid composition is studied using monoclonal antibodies. Differences in surface antigen expression reflects differences between the different subsets of differentiated cells. Comparisons of the surface antigen expression between retinoic acid induced and HMBA induced differentiated cells indicate that differentiation occur along distinctly different differentiation pathways (Andrews *et al* 1990). Further to this, cultures of HMBA induced differentiated EC cells have shown no significant evidence for the production of neuronal cell types, (Andrews *et al* 1986) suggesting that HMBA initiates a separate pathway of differentiation than that induced by retinoic acid. Currently, the identity of the HMBA-induced differentiated cells remains unknown.

- **Human TERA2.SP12 Pluripotent Embryonal Carcinoma Stem Cells**

It is known that there is a wide variation between different clonal lineages of human EC lines. This is noticeable when looking at the fact that different lines have different optimal culture conditions, and also a variable capacity for differentiation. This varying differentiation between EC cell lineages means that different clones may be useful for different areas of research. For example, the NTERA2 sub-line differentiates into a variety of cells including neurons (roughly 10% of the total differentiated population) making it a useful tool for the study of neurogenesis (Przyborski et al 2000). However, under these growth conditions, no evidence of glial development was observed (Andrews 1984). Due to this fact Przyborski (2001) established a new sub clone of the TERA2 cell line termed TERA2.SP12. The idea behind this was that it may have a different capacity for differentiation and could therefore be used as a research tool in other fields of biology. The original TERA2 parent lineage is heterogeneous and contains a minor (1-2%) population of true EC cells and is therefore hard to use as a viable tool for research. Przyborski (2001),

developed a novel strategy to isolate and clone new lineages of human EC cells from mixed cell populations, including the TERA2 lineage, producing the TERA2.SP12 lineage.

In brief, TERA2.SP12 human EC stem cells were derived from the earliest available passage of the original TERA2 parent lineage. Immunomagnetic isolation and single cell selection were used to obtain this new clonal EC line. Suspensions of TERA2 (p15) EC cells were incubated with SSEA-3 antibody, a marker of EC cells. SSEA-3 positive cells were then isolated using direct positive magnetic separation. Isolated cells were immediately resuspended then magnetically separated for a second time to increase purity. The isolated EC cells were again resuspended then single cells were picked at random using a micropipette under a dissecting microscope. The single cells were then transferred to their own well of a tissue culture plate and expanded to form colonies of cloned EC cells. Clone TERA2.SP12 was selected for further analysis. The further analysis of the cells involved using a variety of techniques including cell cytometry, immunocytochemistry, immunofluorescent flow cytometry, northern analysis and western analysis, which all confirmed that the cells were indeed EC cells (Przyborski 2001). TERA2.SP12 EC cells were shown to have the capacity to differentiate in a slightly variable way to existing EC cell lines including TERA2 and NTERA2. TERA2.SP12 EC cells are capable of differentiating into both neuronal and glial lineages (Przyborski 2001), where as the existing lines only appear to differentiate into neurons. TERA2.SP12 human EC cells are currently used to research the formation of human neuronal and glial derivatives in our laboratory.

1.3 Aims of This Investigation

1.3.1 Embryonal Carcinoma Stem Cells as a Model for Cell Fate Determination in the Human Ectoderm

As this overview of EC cells has demonstrated, EC cells are very useful tools as cell-based systems for the study of embryogenesis in vitro. Whilst it has been extensively documented that cultured EC cell populations can be induced to form neural cell types, such as with retinoic acid, there is little evidence for the formation of epidermal derivatives. As a result of this I propose to investigate the potential of TERA2.SP12 EC stem cells to form neural AND epidermal tissues under different culture conditions. Previous studies in our laboratory have produced preliminary data indicating the potential for epidermal differentiation by TERA2 cultures. For example, a large-scale microarray study revealed that cultures of human EC cells formed neural cell types and also expressed some epidermal markers under alternative growth conditions (Przyborski et al 2001, 2003). When EC cell populations are exposed to HMBA the expression of high levels of mRNA for keratin, fibronectin and BMPs associated with epidermal differentiation are seen. I aim to extend these earlier observations and develop a model system that can be used to research the differentiation of the human ectoderm along neural and epidermal pathways. Subsequent to this, it would also be of value to more fully characterise the identity of the differentiated end products produced when EC cells are exposed to HMBA as this is currently unknown and there is very little evidence of neural differentiation in these cultures.

The characterization of two alternative pathways of ectodermal differentiation will be of value to the study of the fundamental processes of cell fate determination in the human ectoderm. This could lead to the potential development of a culture system able to study

the epidermal differentiation from pluripotent stem cells along side the existing culture system used to study neural development by human EC cells. These two systems could be advantageous to the study of molecular mechanisms specifically involved in certain pathways of differentiation, for example, the involvement of molecules that are known to suppress the formation of neural derivatives.

Chapter 2:

Experimental Approach

2.1 Cell Culture:

Human TERA2.SP12 EC stem cells were maintained at high density as previously described (Przyborski 2001). Briefly, to retain an optimum EC cell phenotype TERA2.SP12 cells were cultured in tissue culture flasks (Nunc) using Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% foetal calf serum and 2mM L-glutamine under a humidified atmosphere of 5% CO_2 in air at 37°C. TERA2.SP12 cells were passaged when fully confluent, every 3-4 days, using glass beads and underwent a 1:3 split. The medium was changed twice weekly.

TERA2.SP12 EC cells were induced to differentiate with either the introduction of retinoic acid (10μM) (Sigma) or HMBA (3mM) (Sigma) into the media. In preparation for differentiation, confluent monolayers of TERA2.SP12 EC cells were treated with 0.25% trypsin in versene solution (1000ml Phosphate buffered saline with 0.2g EDTA and 3ml 0.5% phenol red solution) for 2/3 minutes to produce a single cell solution. The suspended stem cells were counted using a haemocytometer and then seeded at $1.5x10^6$ cells per T75 tissue culture flask in DMEM containing either retinoic acid or HMBA using the concentrations stated above. The media containing the differentiation inducing agent was changed twice weekly. Cultures treated with retinoic acid and HMBA were maintained for up to 4 weeks.

RNA and protein were harvested from cells after 1,2,and 4 weeks of differentiation. In brief, differentiated cells were harvested with 0.25% trypsin/versene solution to obtain a single cell suspension. The cells were counted then spun down using a centrifuge. The cell pellet was resuspended in sterile phosphate buffered saline (PBS) then stored in Eppendorf tubes of known cell numbers at –80 °C until required.

TERA2.SP12 stem cells and their retinoic acid and HMBA induced derivatives were also grown on glass coverslips (16mm diameter). Cultures of the TERA2.SP12 stem cells were trypsinised and counted as described above, and $0.2x10^6$ cells were suspended in 4ml of media per well on a six well plate, each well containing 2 coverslips. Poly-D-lysine (0.1μg/ml) was used to coat the glass coverslips overnight prior to seeding cells. Retinoic acid (10μM) or HMBA (3mM) was added to the wells to induce differentiation as before. The media was changed twice weekly and the cells incubated with the agents for up to 4 weeks.

TERA2.SP12 stem cells were also grown as aggregates in bacteriological dishes (VWR International). Cultures of the TERA2.SP12 stem cells were trypsinised and counted as described above, and $1.5x10^6$ cells were suspended in 25ml of DMEM media plus either retinoic acid (10μM) or HMBA (3mM) per plate. Medium was changed every 3 1/2 days and the cells were suspended in new bacteriological dishes each time. After an incubation period of 3 weeks the aggregates were either harvested for RNA and protein as previously described, or were seeded onto coverslips coated with poly-d-lysine also described above. The media was changed twice weekly and the cells were incubated with the agents for a further 3 weeks.

2.2 Analysis of Cell Surface Antigen Expression: Flow Cytometry:

Flow cytometry was used to analyse the changes in cell surface antigen expression before and after the differentiation of TERA2.SP12 cells in response to either retinoic acid or HMBA as described. The expression of cell surface antigens has been used previously to indicate the status of cellular differentiation by human pluripotent TERA2.SP12 stem cells (Przyborski 2001).

Table 2.1:

Antibodies used for cytometric analysis of TERA2.SP12 stem cells and their differentiated derivatives. *All antibodies were diluted as indicated in wash buffer (PBS (without Ca^{2+}/Mg^{2+}) and 0.1% bovine serum albumin (BSA) and 0.1% sodium azide, pH 7.0). Primary antibodies were generously provided by Prof. P. Andrews, University of Sheffield, whilst the secondary antibody was obtained from Cappell, USA.*

Primary antibodies			
IgG	P3X (1:5)	IgM	P3X (1:5)
	SSEA-4, a globoseries glycolipid antigen (1:2)		SSEA-3, a globoseries glycolipid antigen (1:5)
	VINIS-53, recognises NCAM (1:5)		TRA-1-60, a keratan-sulphate-associated glycoprotein antigen (1:5)
	Me311, a ganglioseries antigen (1:2)		A2B5, a ganglioseries antigen (1:2)
Secondary antibodies			
	α-mouse-IgG-FITC (1:20)		α-mouse-IgM-FITC (1:20)

Procedure:

1) 1ml 0.25% trypsin/versene solution was used to create a single cell suspension of EC cells or their differentiated derivatives. Cell number was determined using a haemocytometer.
2) Suspended cells were pelleted and resuspended in wash buffer. $0.2x10^6$ cells in a 200μL volume was added to the appropriate number of wells in a 96 well plate. The plates were lightly centrifuged and the supernatant removed, resulting in a cell pellet in the bottom of each well.
3) 50μl of primary antibody (see Table 2.1) was incubated with the pellets for 1 hour at 4°C.
4) The primary antibody was removed with 3 washes in wash buffer.
5) 50μl of secondary antibody (see Table 2.1) was incubated with the cells for 30 minutes at 4 °C.
6) The secondary antibody was removed with 3 washes in wash buffer.
7) Cell pellets were resuspended in 500μl of wash buffer with 10μl of propidium iodide (0.25mg/ml, diluted 1:50 for use), to pick out any dead or dying cells when run through the flow cytometer.
8) The samples were passed through the flow cytometer (Coulter XL) and cytometric traces were recorded for each of the primary antibodies tested. Thresholds determining the number of positively expressing cells were set against the negative control P3X before levels of specific cell surface antigen were determined.
9) Viable, healthy cells were selected by the cytometer for investigation to eliminate false positive or negative results. This was done by the selection of cells that were the correct diameter, 10μm, the idea being to avoid cell debris. These cells were

gated then from these gated cells, cells were selected that were propidium iodide positive for reasons stated above.

10) Cytometric analysis was carried out on three separate batches of cultured cells that were treated separately as individual experiments.

2.3 Protein Expression Profiling Using Western Blot:

Protein samples were harvested from TERA2.SP12 stem cells and their differentiated derivatives in preparation for western blot analysis.

Table 2.2

Antibodies used for western analysis of protein expression in TERA2.SP12 stem cells and their differentiated derivatives: *All antibodies were diluted in blocking buffer (5% milk powder in TBS (Tris buffered saline) (10X solution: 6.1g tris base, 43.8g NaCl, 500ml H_2O, pH 7.5) and 0.02% tween).*

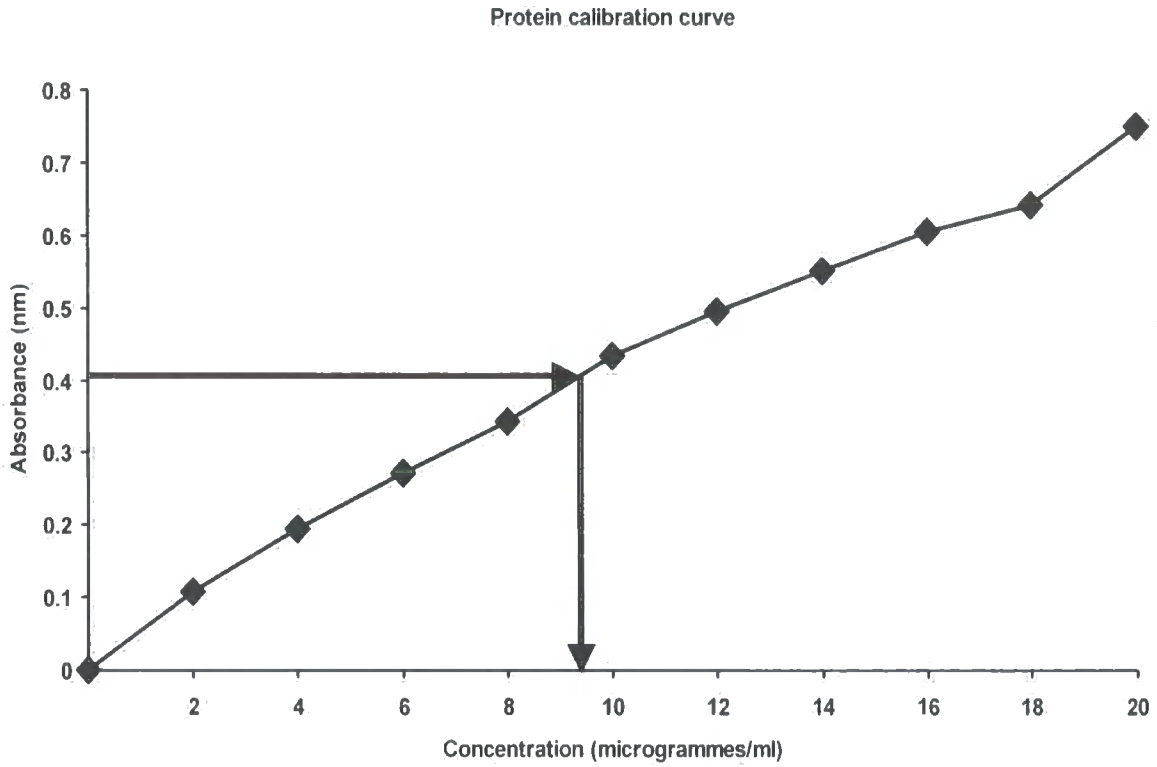
Primary Antibody	**Clone**	**Isotype**	**Dilution factor**
β-actin (Sigma)	AC-15	Mouse IgG	1:1500
NF-68 (Sigma)	NR4	Mouse IgG	1:2000
NSE (Chemicon)	-	Mouse IgG	1:2000
Keratin (Chemicon)	-	Mouse IgG	1:500
Cytokeratin-8 (Sigma)	M20	Mouse IgG	1:7000
Secondary Antibody	**Clone**	**Isotype**	**Dilution factor**
Anti-mouse Ig-horse radish peroxidase	-	Mouse IgG	1:1000

Procedure:

1) Protein samples were lysed in igepal lysis buffer (for 1ml: 10µl igepal (Sigma) 50µl 1M tris (pH8), 50µl 3M NaCl, 1µl $MgCl_2$, 896µl dH_2O) containing protease inhibitors (Complete mini protease inhibitor cocktail, Roche Molecular Biochemicals). The resulting protein solutions were centrifuged at 15000 rpm for 3 minutes and the supernatant transferred to a fresh tube.
2) Protein concentrations were determined using the Bradford-based assay. 2µl of sample was added to 798µl dH_2O and 200µl of the BioRad solution. The concentrations were calculated against a standard calibration curve which had been set up using BSA standards using the UV spectrometer (see Figure 2.1). The volumes of the samples were then adjusted according to requirement. 12µg of sample was diluted in 12µl of loading buffer (5X stock: 2.5ml 1M Tris pH 6.8, 5ml glycerol, 1g SDS, 0.05g bromophenol blue, 2.5ml 2β-mecapto-ethanol) per well on the polyacrylamide gel.
3) SDS-polyacrylamide gels were then prepared using the BioRad minigel system. 10% acrylamide gels were used as the separating gel since it was concluded the most appropriate density to study the separation of the particular proteins of interest.
4) A 4% acrylamide stacking gel was subsequently poured and polymerised on top of the separating gel.
5) The protein samples (1µg/µl) suspended in the loading buffer were denatured for 3 minutes at 96°C.
6) The samples were loaded onto the gel and electrophoresed at 200v for 35-45 minutes until the dye front had reached the bottom of the gel.

Figure 2.1.

Graph of the protein standards used to determine concentration of the protein samples from TERA2.SP12 cells and their differentiated derivatives. *Protein concentrations (μg/ml) were calculated using a standard curve made up using BSA protein standards. The readings were taken off the graph after absorbance had been determined (arrows). The calibration curve was taken from the UV spectrometer.*



7) The separated proteins were then transferred to PVDF membrane (Amersham) by electroblotting at 100v for 1 ½ hours in towbin transfer buffer (3.03g tris-base, 14.4g Glycine, 200ml methanol and 800ml dH_2O) using the BioRad mini gel transfer apparatus.

8) PVDF membranes (Amersham) were subsequently stained with Ponceau S solution (0.2% Ponseau S in 5% acetic acid) to check the quality of the transfer. The membranes were then left to dry.

9) Rehydration of the membrane is required according to the manufacturers guidelines before it was blocked in blocking buffer (5% milk powder in TBS (10X solution: 6.1g tris base, 43.8g NaCl, 500ml H_2O, pH 7.5) and 0.02% tween) for 1-2 hours at room temperature.

10) The membrane was then incubated with the primary antibody (see Table 2.2), diluted in blocking buffer, for 1 hour at room temperature on a bench top rocker. (NB: Primary antibody titrations was carried out in all cases to determine optimal antibody dilution – see below)

11) Primary antibody was removed by 3 washes in blocking buffer. Membranes were subsequently incubated with the secondary antibody (see Table 2.2), also diluted in blocking buffer, for a further 60 minutes at room temperature.

12) Membranes were washed 3 times in TBS, followed by a final PBS wash.

13) Antibody-antigen complexes were detected using chemiluminescence according to the manufacturers guidelines (Amersham). The luminescence signal was detected by film photography (Hyperfilm ECL, Amersham).

14) The intensity of the bands was analysed by routine densitometry using the BioRad Model GS-690 Imaging Densitometer.

15) All results were reproduced using a second batch of cells grown independently.

Titration of Primary Antibodies: A titration blot was carried out if the dilution factor of the primary antibody had not been predetermined. Gels were run containing 3 sets of the same samples, then various dilutions of the antibody were incubated with duplicate strips of membrane to determine which dilution factor obtained optimum results (see Figure 2.2). Then a full set of samples was run on a gel as described above and incubated with the determined dilution of antibody.

Figure 2.2

Typical example of a titration blot: *The primary antibody was diluted 1:500, 1:1000 and 1:2000, then incubated on the strips of membrane containing the equal loading of protein samples. Bands were seen to decrease in intensity with an increase of antibody dilution. In this instance, a dilution factor of 1:1500 was used for examining protein expression by western analysis.*

1:500 1:1000 1:2000

2.4 Immunocytochemistry:

Cell cultures grown on glass coverslips were used for immunocytochemistry.

Table 2.3

Antibodies used to examine protein expression by immunocytochemistry: *(All antibodies are diluted in staining buffer)*

Antibody	**Dilution factor**
Laminin (Sigma)	1:1000
Fibronectin (Sigma)	1:1000
NF-68 (Sigma)	1:400
β-III-tubulin (Sigma)	1:400
Cytokeratin-8 (Sigma)	1:500
Keratin-14 (Chemicon)	1:500
Secondary Antibody	**Dilution factor**
Anti-mouse IgG FITC (Sigma)	1:80

Procedure:

1) Cells grown on coverslips were either fixed in methanol or paraformaldehyde (PFA) according to the method for optimal antigen detection.

   Methanol-fixed: Cells were rinsed in PBS, then fixed in ice-cold methanol for 10 minutes and stored along with a desiccant at –80°C until required.

   PFA-fixed: Cells were incubated with PFA (2% PFA in PBS) for 20 minutes then rinsed for 2 minutes in 3X PBS. Cells were dehydrated for 2 minutes consecutively in PBS, H_2O, 50% ethanol, 70% ethanol, 95% ethanol then finally 100% ethanol and stored at -80°C with a desiccant until required.

2) For immunocytochemistry, cells were incubated in blocking buffer (1% BSA in PBS with 0.2% tween) for 1-2 hours at room temperature.
3) The primary antibody (see Table 2.3) was then added, diluted in staining buffer (0.1% BSA in PBS with 0.2% tween), and left to incubate for 1 hour.
4) The primary antibody was removed with 3 washes of 5 minutes each with staining buffer.
5) The coverslips were then incubated with the secondary antibody (see table 2.3), diluted in staining buffer, for 1 hour at room temperature.
6) The secondary antibody was then removed by a further 3 washes of staining buffer.
7) The coverslips were then mounted onto microscope slides using Vectashield (Vector Labs), an aqueous mountant.
8) Stained coverslips were examined using a fluorescence microscope (Axiovert 135) using the x5, x10, x20, and x32 objectives. Phase contrast and their corresponding fluorescence images were collected using a combination of a digital camera (Nikon E5000) and standard 35mm film camera (Nikon).
9) All results were reproduced using a second batch of cells grown independently.

2.5 Analysis of Gene Transcription by RT-PCR:

RT-PCR was used to detect the expression of certain genes. RNA samples were taken from cultures of TERA2.SP12 stem cells, their differentiated derivatives and differentiated aggregates grown on coverslips. RNA samples were used for RT-PCR analysis using specifically designed PCR primers.

Table 2.4:

List of PCR primers designed and used for RT-PCR analysis. *All primers were manufactured by MWG, Germany.*

Human Gene	Accession Number	Optimal Annealing Temperature (°C)
β-actin	BC009275	55.0
TLE1	M99435	59.4
TLE2	M99436	62.0
BMP4	M22490	61.0
BMP6	M60315.1	54.8
GATA-1	NM_002049.1	61.3
MSX1	NM_002448	60.2
SMAD1	BC001878	54.7
SMAD4	NM_005359.2	48.9
SMAD6	NM_005585.2	63.6
SMAD7	NM_005904.1	62.7

Table 2.5

List of appropriate PCR primer sequences and product size.

Human Gene	Primer Sequence		Product size (bp)
β-actin	For	ATCTGGCACCACACCTTCTACAATGAGCTGC	803
	Rev	CGTCATACTCCTGCTTGCTGATCCACATCTG	
TLE1	For	TCATCGGGCAGCAGCAGTTG	450
	Rev	CATTTTCCCGGGGCGAGTTGG	
TLE2	For	CGGGGCTGCTTGCTCTGTCT	442
	Rev	GTGCTGGCGGGAAGGTCATT	
BMP4	For	ACCCGGGAGAAGCAGCCAAACTAT	553
	Rev	AGCGGCACCCACATCCCTCTACTA	
BMP6	For	TGCCGGCTCTAGTACCTTTTCAGT	430
	Rev	GCCCGCCCACACCACGAC	
GATA-1	For	ACGACACTGTGGCGGAGAAATG	439
	Rev	TGAGCGGAGCCACCACAGTAGT	
MSX1	For	GTGGCGCCCGTGGGACTCTACA	443
	Rev	CACCGATTTCTCTGCGCTTTCTTG	
SMAD1	For	AAGCGTTCCATGCCTCCTC	411
	Rev	CTCCCCAGCCCTTCACAAA	
SMAD4	For	ATCCCTTAAAATTACCAGACAA	462
	Rev	TGCCCCAAGATACAAAAAC	
SMAD6	For	CGTGGGCCGCCTCTATGC	480
	Rev	CGCCGGGGCCGCCACTATCT	
SMAD7	For	AGGAGCGGCAGATGGGGAGACAAC	453
	Rev	AGAGGCTGAGGGGAGAGGGCACTG	

Procedure:

1) Cell pellets were lysed and RNA extracted using TRI reagent (Sigma) following the instructions outlined by the manufacturer. 1ml of TRI reagent was used to lyse 5-10x10^6 cells by repeated pipetting. Samples were then left at room temperature for 5 minutes. 0.2ml of chloroform was added then samples were shaken for 15 seconds before standing at room temperature for 7 minutes. The resulting mixture was centrifuged at 12000g for 15 minutes at 4 °C. The colourless upper aqueous phase was transferred to a fresh tube and 0.5ml of isopropanol was added and the mixture allowed to stand for 7 minutes. The samples were centrifuged at 12000g for 10 minutes at 4°C. The RNA precipitate formed a pellet on the side and bottom of the tube. The supernatant was removed and the RNA pellet washed by the addition of 1ml of 75% ethanol. The mixtures are then centrifuged at 7500 Xg for 5 minutes at 4°C. The supernatant was removed and the pellets air dried for 5-10 minutes. 50µl of ddH_2O was added then the pellets stored at –20°C.
2) Final preparations of total RNA samples were read on the UV spectrometer (Fisher) to determine the total yield and purity of the RNA. Total yield was calculated using the following equations. (RNA samples diluted in H_2O):

Concentration of RNA sample = 40x A_{260} x dilution factor

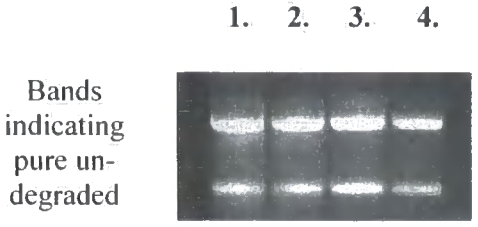
Total yield = concentration x volume of sample in millilitres

The purity was calculated from the ratio of the readings at 260 nm and 280nm (A_{260}/A_{280}). These calculations are measured with the RNA samples diluted in 10mM Tris-Cl pH 7.5. Pure RNA samples should have a ratio of 1.9-2.1.

3) 10μg samples of pure total RNA were run down a 1% RNAase-free agarose gel (1% agarose in TBE running buffer and 0.02% ethidium bromide) to check for RNA degradation. The resulting images of the RNA bands was obtained by using a transilluminator (Gel Doc 2000, BioRad). An example is shown in Figure 2.3.

Figure 2.3.

Typical example of pure non-degraded total RNA. *Non-degraded total RNA samples have two distinct bands representing 28s and 18s ribosomal RNA. Smearing of these bands down the lane would indicate degradation of total RNA.*



1. EC cell sample **2.** EC cells differentiated with retinoic acid after 4 weeks incubation
3. EC cells differentiated with HMBA after 4 weeks incubation **4.** Neurosphere

4) Total RNA samples are reverse transcribed using the oligo-dT (15) primer (Promega). 1μl of the primer was combined with the RNA sample and H_2O was added to 25μl. The resulting mixture is heated at 70°C for 10 minutes then ice cooled for a further 10 minutes. To each reaction was added 8μl x5 buffer, 1μl 0.1M DTT, 5μl 10mM dNTP, 1μl RNase H-RT, and 1μl RNasin (All Promega). Each mixture is incubated at 37°C for 2 hours, denatured at 95°C for 5minutes then left to cool on ice. The resulting cDNA samples can then be stored at -20°C until required.

5) PCR primers were designed using the Primer Select computer package (DNAStar) and purchased from MWG. Newly synthesized primers were diluted with ddH_2O and used at a concentration of 20 pmol/ml.

6) For the polymerase chain reaction, the following reagents were added to each reaction tube and overlaid with mineral oil:

PCR Master mix (Promega)	6.25µl
5' Primer	0.5µl
3' Primer	0.5µl
cDNA sample	1.5µl
ddH_2O	3.75µl
Total	12.5µl

The following PCR reactions were then carried out using a Perkin Elmer thermal cycler:

94°C	3 minutes	1 cycle
94°C	1 minute	35-40 cycles
Optimal annealing temperature (see Table 2.4)	1 minute	
72°C	2 minutes	
72°C	10 minutes	1 cycle

7) The PCR products were separated from the mineral oil then run down a 1% agarose gel as mentioned above. Again the DNA bands were visualised using the transillunimator (Gel Doc 2000, Bio Rad) and a printed record obtained.

8) All results were reproduced using a second batch of cells grown independently.

Chapter 3

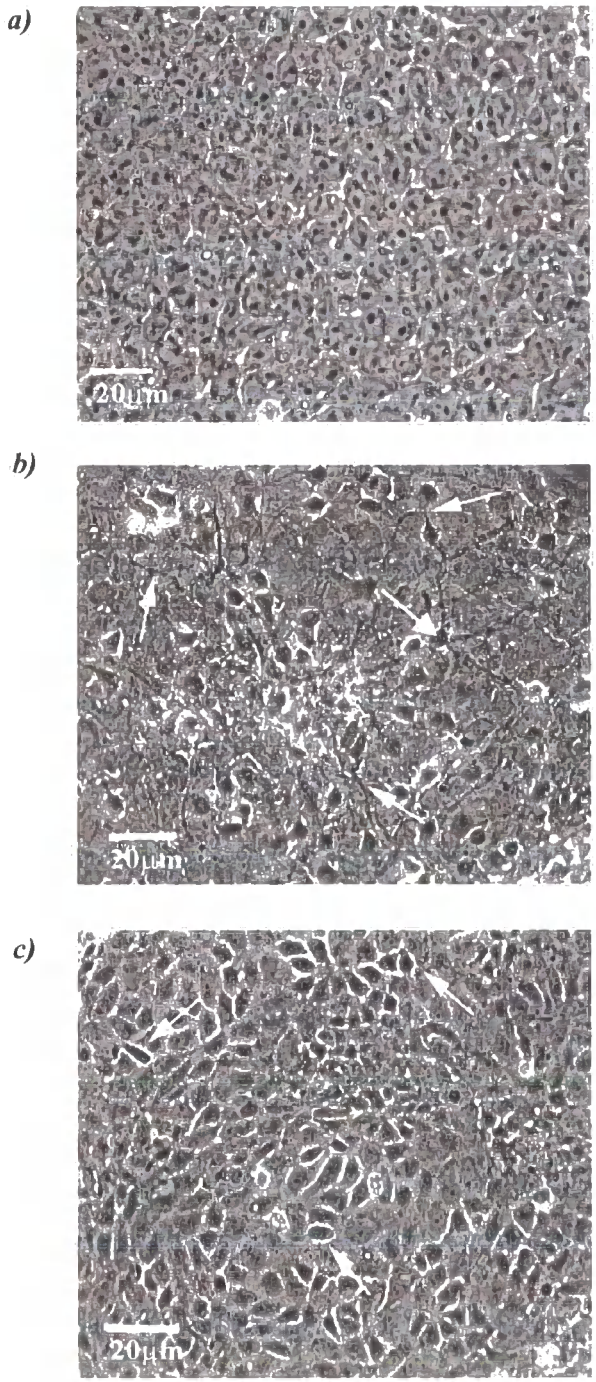
Human Embryonal Carcinoma Cells as a Model of Cell Fate Determination

3.1 Differentiation of TERA2.SP12 Embryonal Carcinoma Cells In Vitro

Cultures of TERA2.SP12 EC stem cells were maintained at high confluency to maintain their pluripotent phenotype. In this state all cells remain undifferentiated and had a high nuclear to cytoplasmic ratio (Figure 3.1a). TERA2.SP12 EC stem cells were induced to differentiate by introduction of either retinoic acid (10μM) or HMBA (3mM) into the culture media. Differentiated cells were morphologically identified after just 2-3 days. In response to the addition of retinoic acid, TERA2.SP12 EC cells differentiated into a heterogeneous culture consisting of numerous cell types. Four weeks after introduction of the retinoic acid, terminally differentiated neurons expressing neurites were clearly visible by phase photography (Figure 3.1b). In response to HMBA, TERA2.SP12 EC cells differentiated into cells that displayed a large flat phenotype that was seen throughout the entire culture (Figure 3.1c). Many of these large regular cells had a distinct phase bright outline, which may indicate the presence of extra-cellular material. In general, there was almost a complete lack of any visible neural cell types within the HMBA-induced cultures, even after 4 weeks of incubation. Very rarely there appeared to be 1 or 2 neurons observed in older cultures. This differs significantly from when the EC cells were differentiated with retinoic acid, since

roughly 10-20% of the cells in retinoic acid-induced cultures formed morphologically identifiable neurons. Based on their morphological phenotype, there appeared to be a lot of similarity between the phase-bright non-neuronal cells observed in both retinoic acid and HMBA-induced cultures.

FIGURE 3.1: ***Phase contrast images of TERA2.SP12 human EC cells and their differentiated counterparts.*** ***a)*** *TERA2.SP12 EC stem cells were maintained at high confluency. Note the high nuclear to cytoplasmic ratio.* ***b)*** *TERA2.SP12 EC cells after 4 weeks exposure to 10μM retinoic acid. Cells have differentiated into a heterogeneous culture consisting of numerous cell types including neurons expressing neurites (arrows). Non-neural cells were observed beneath the neuronal cell types.* ***c)*** *TERA2.SP12 EC cells after 4 weeks exposure to 3mM HMBA. Differentiated cells have a large flat phenotype with a phase bright outline (arrows) which is consistent throughout the culture.*



3.2 Regulation of Cell Surface Protein Expression during TERA2.SP12 Differentiation:

Differentiation of TERA2.SP12 stem cells was monitored using flow cytometry. Antibodies against cell surface markers specific for stem cells (SSEA-3, SSEA-4, globoseries glycolipid antigens, and TRA-1-60, a keratan-sulphate-associated glycoprotein antigen) and markers for terminal neuronal differentiation (A2B5, a ganglioseries antigen, VINIS-56, which recognises NCAM, and Me311, also a ganglioseries antigen) were used to monitor protein expression. For each antibody used, graphical traces were obtained showing the intensity and quantity of fluorescence from which the percentage of positively stained cells within the sample was calculated (Figures 3.2-3.4, 3.6-3.8). From these data, graphs showing the profiles of protein expression during EC cell differentiation were produced (Figures 3.5 and 3.9). The percentage of EC cells expressing the markers SSEA-3, SSEA-4 and TRA-1-60 before differentiation was 90%, 98% and 98% respectively. Cytometric data clearly indicates a down regulation of the stem cell markers when the TERA2.SP12 EC cells are exposed to either retinoic acid or HMBA. After only 1 week exposure to retinoic acid the proportion of cells expressing the markers had decreased to 10%, 96% and 30% and by week 4 were down to 2%, 2% and 3% respectively (Figure 3.5). After 1 week incubation with HMBA the percentage of positively fluorescing cells was 19%, 99% and 90% respectively, and by week 4 this had decreased to 2%, 11% and 6% (Figure 3.5). From these data it can be seen that the stem cell markers appear to be down regulated by cells differentiating in response to HMBA at a significantly slower rate than with those exposed to retinoic acid. However, in both cases the stem cell markers are almost completely absent by 4 weeks of differentiation.

In contrast, proteins recognised by antibodies A2B5 and VINIS-56 were up-regulated during retinoic acid- and HMBA-induced differentiation of TERA2.SP12 cells (Figure 3.9). These

markers were detected at very low levels on the EC stem cells (4% A2B5 and 6% VINIS-56). After 4 weeks exposure to retinoic acid both A2B5 and VINIS-56 were up-regulated to 97%, indicating that the TERA2.SP12 cells had responded to the inducing agent. The cell surface markers A2B5 and VINIS-56 are also thought to be associated with detecting the neural lineage (Eisenbarth et al 1979, Andrews et al 1996). Accordingly, the up-regulation of A2B5 and VINIS-56 appears to correlate with the differentiation of identifiable neurons. In contrast, after 4 weeks incubation with HMBA the level of A2B5 and VINIS-56 expression had increased by 17% and 31% respectively. The expression of A2B5 and VINIS-56 in HMBA induced cultures is significantly less than in retinoic acid treated cultures, which may reflect their different capacities for neuronal differentiation. The ganglioseries cell surface antigen recognised by Me311 showed minimal regulation by either inducing agent, indicating that during these two distinct pathways of differentiation an up-regulation of this antigen is not induced.

Figure 3.2: ***Cytometric traces showing the expression of cell surface markers.*** *Traces showing the change in expression of the stem cell marker SSEA-3 after differentiation of TERA2.SP12 EC cells with either retinoic acid (10μM) or HMBA (3mM) over a period of 4 weeks. Percentage value indicates the proportion of cells positively expressing SSEA-3. (x-axis=Intensity of cells expressing SSEA-3, y-axis=number of cells flourescing)*

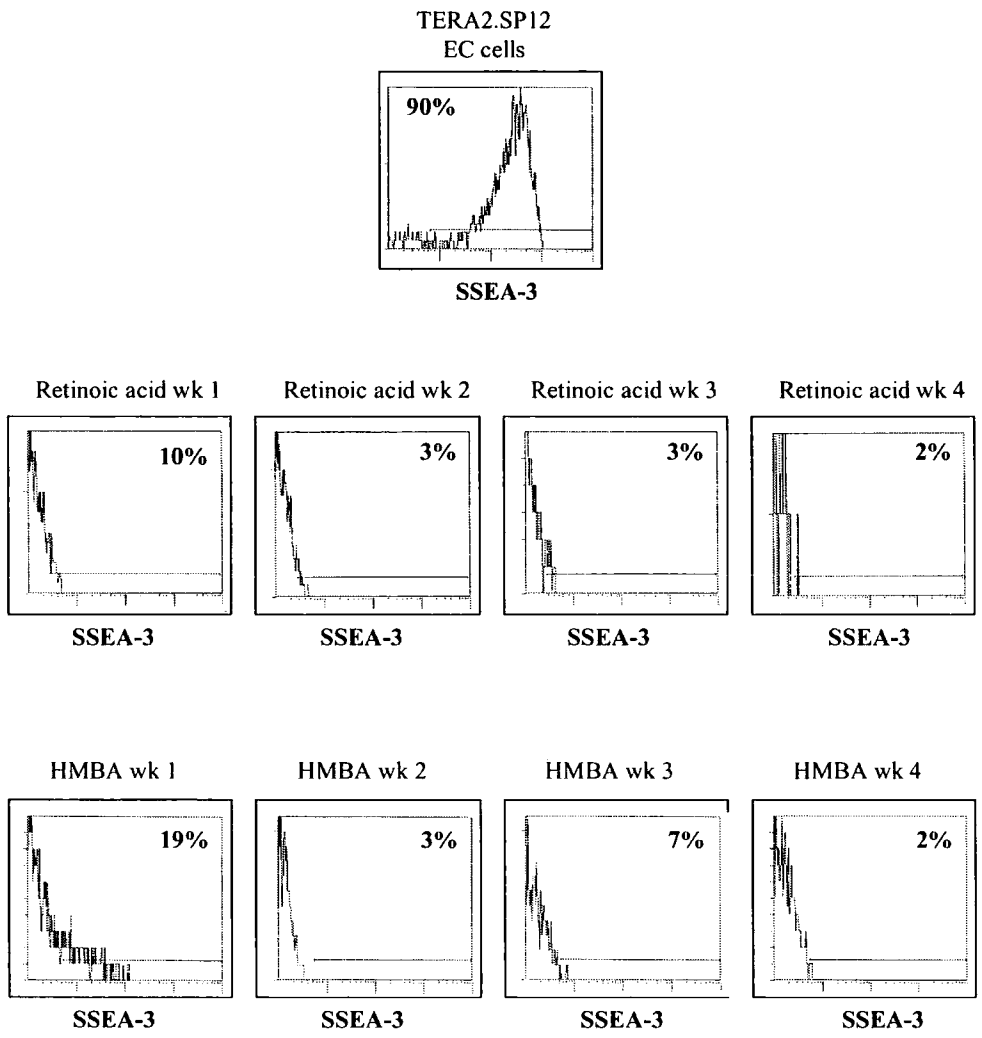


Figure 3.3: *Traces showing the change in expression of the stem cell marker SSEA-4 after differentiation of TERA2.SP12 EC cells with either retinoic acid (10μM) or HMBA (3mM) over a period of 4 weeks. Percentage value indicates the proportion of cells positively expressing SSEA-4. (x-axis=Intensity of cells expressing SSEA-4, y-axis=number of cells flouresecing)*

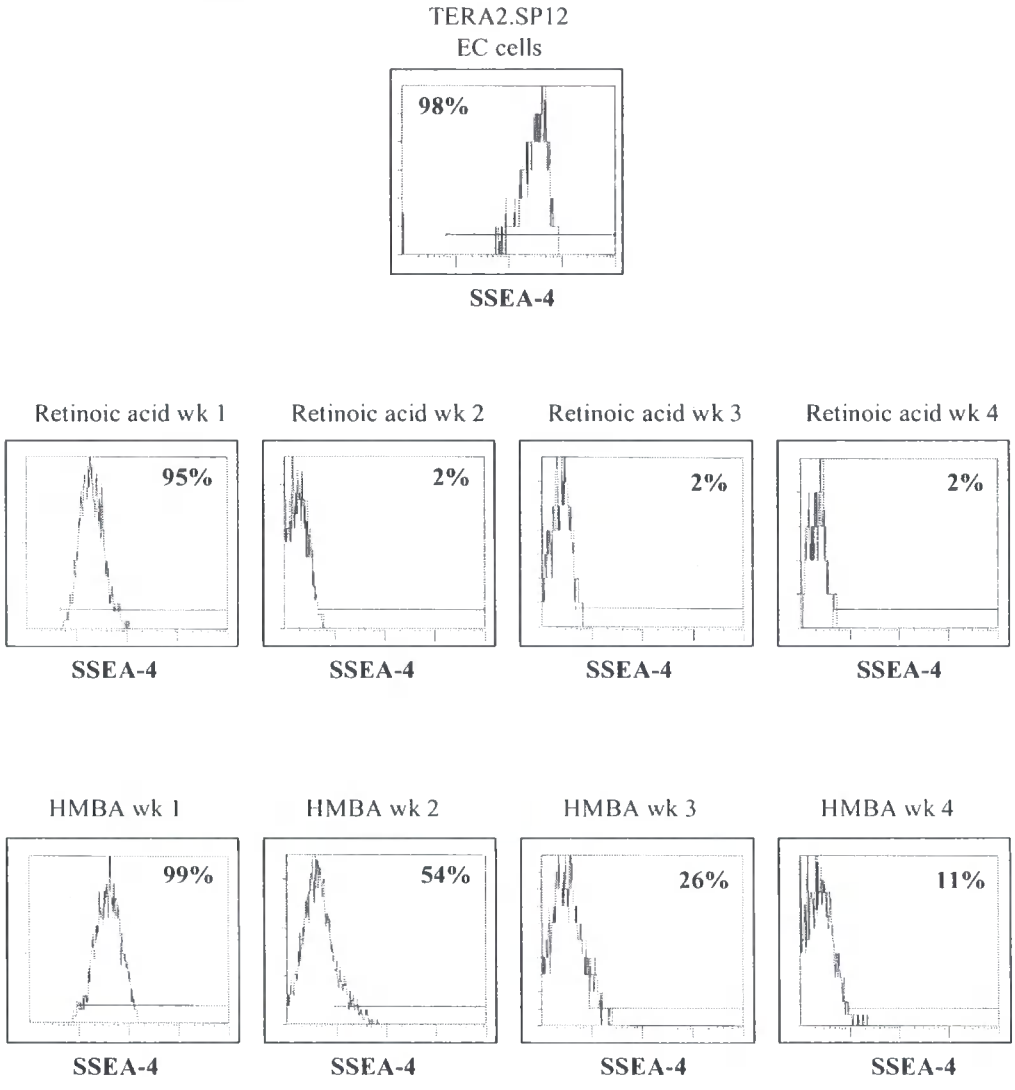


Figure 3.4: *Traces showing the change in expression of the stem cell marker TRA-1-60 after differentiation of TERA2.SP12 EC cells with either retinoic acid (10μM) or HMBA (3mM) over a period of 4 weeks. Percentage value indicates the proportion of cells positively expressing TRA-1-60. (x-axis=Intensity of cells expressing TRA-1-60, y-axis=number of cells flourescing)*

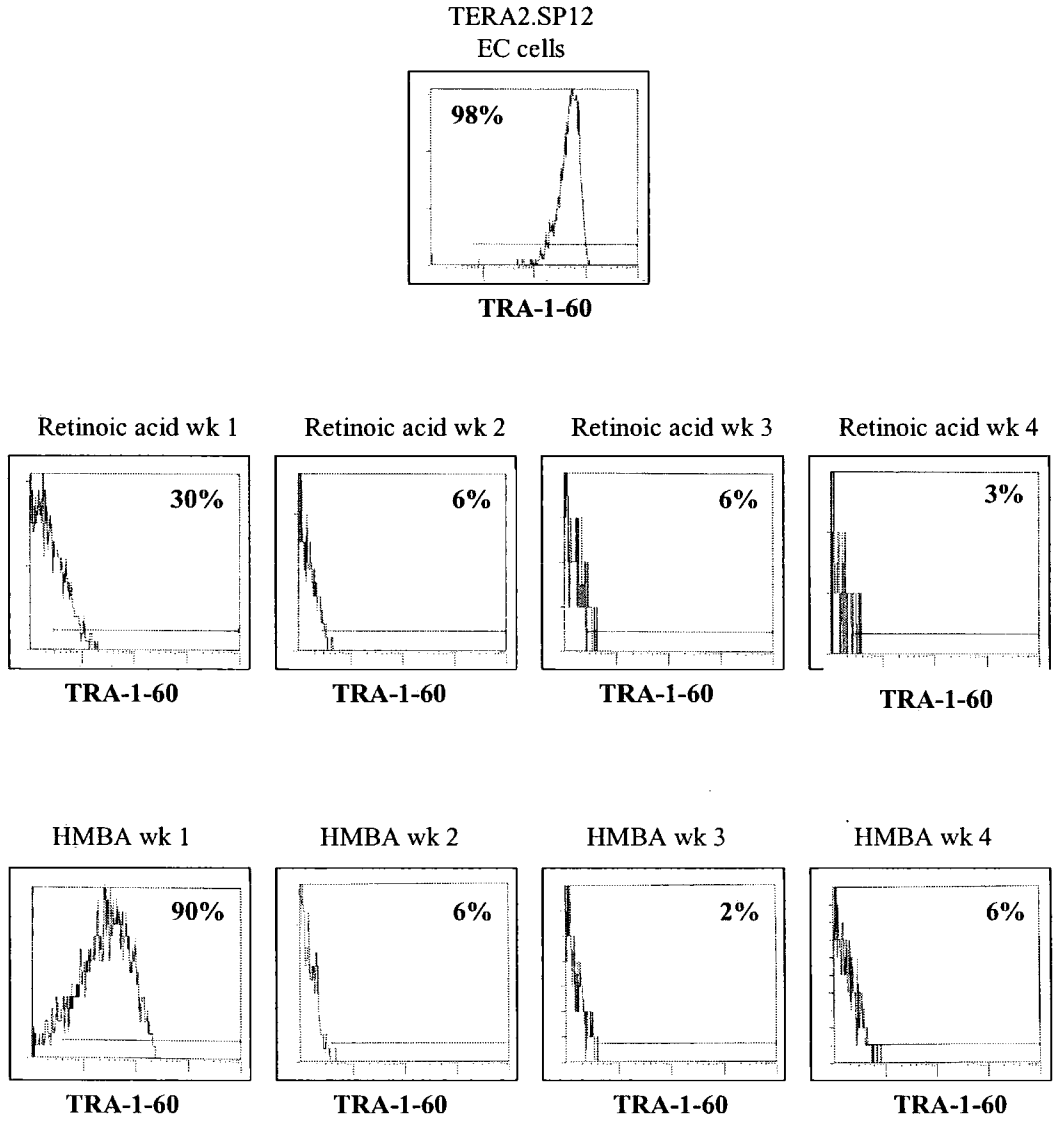
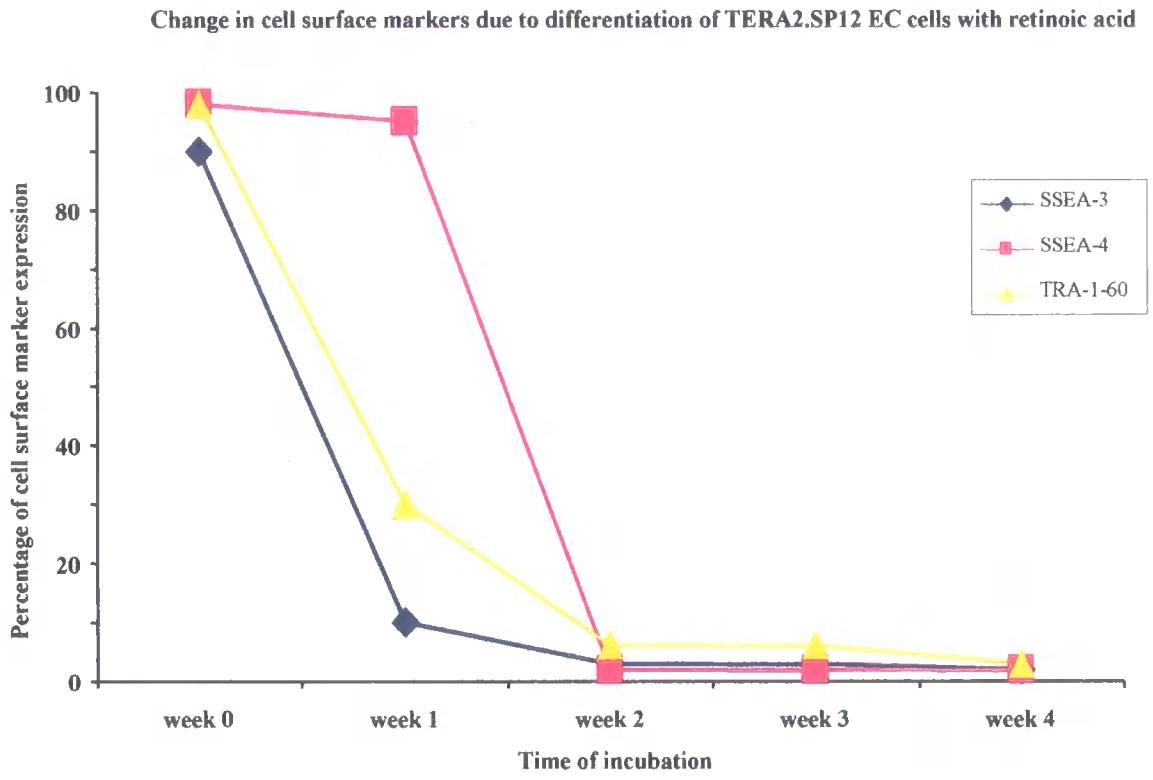


FIGURE 3.5: ***Overview of the expression of cell surface stem cell markers during cellular differentiation.*** *TERA2.SP12 EC cells were exposed to either a) retinoic acid (10μM) or b) HMBA (3mM). Data were determined by flow cytometry. These figures represent typical data trends seen in all 3 separate data sets.*

a)



b)

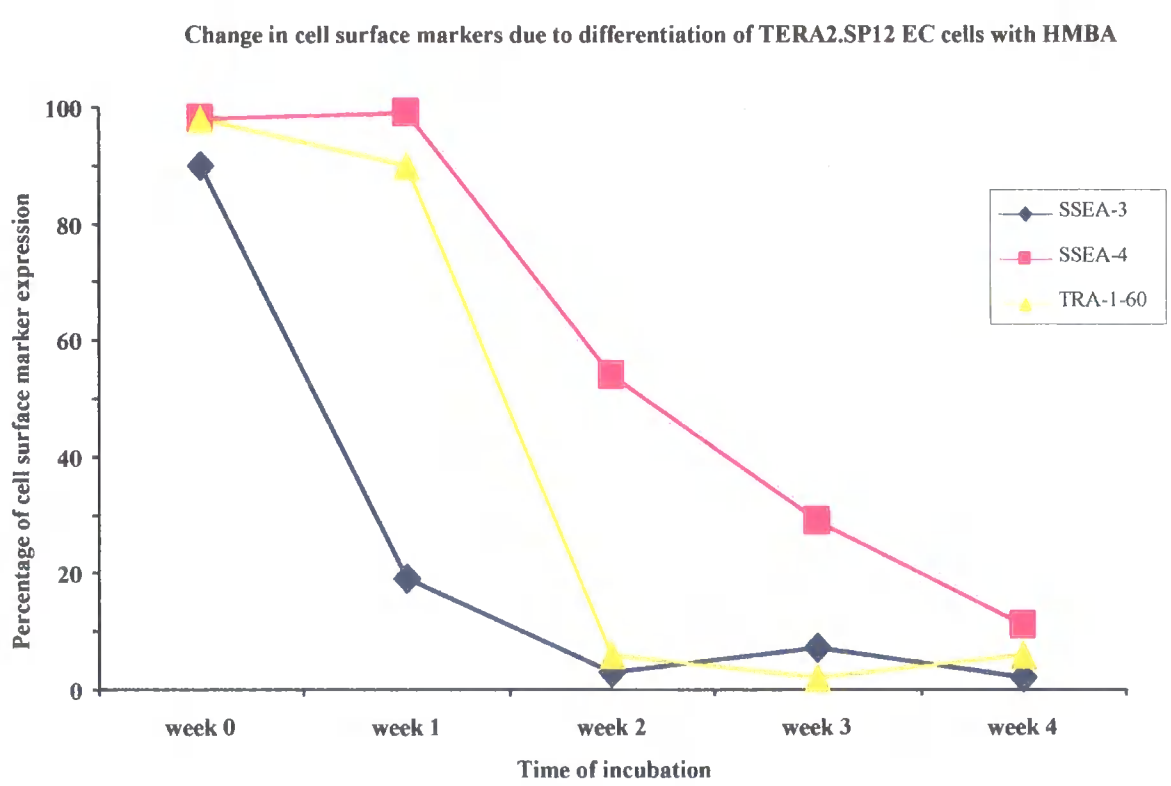


Figure 3.6: *Traces showing the change in expression of the differentiation marker A2B5 after differentiation of TERA2.SP12 EC cells with either retinoic acid (10μM) or HMBA (3mM) over a period of 4 weeks. Percentage value indicates the proportion of cells positively expressing A2B5. (x-axis=Intensity of cells expressing A2B5, y-axis=number of cells flourescing)*

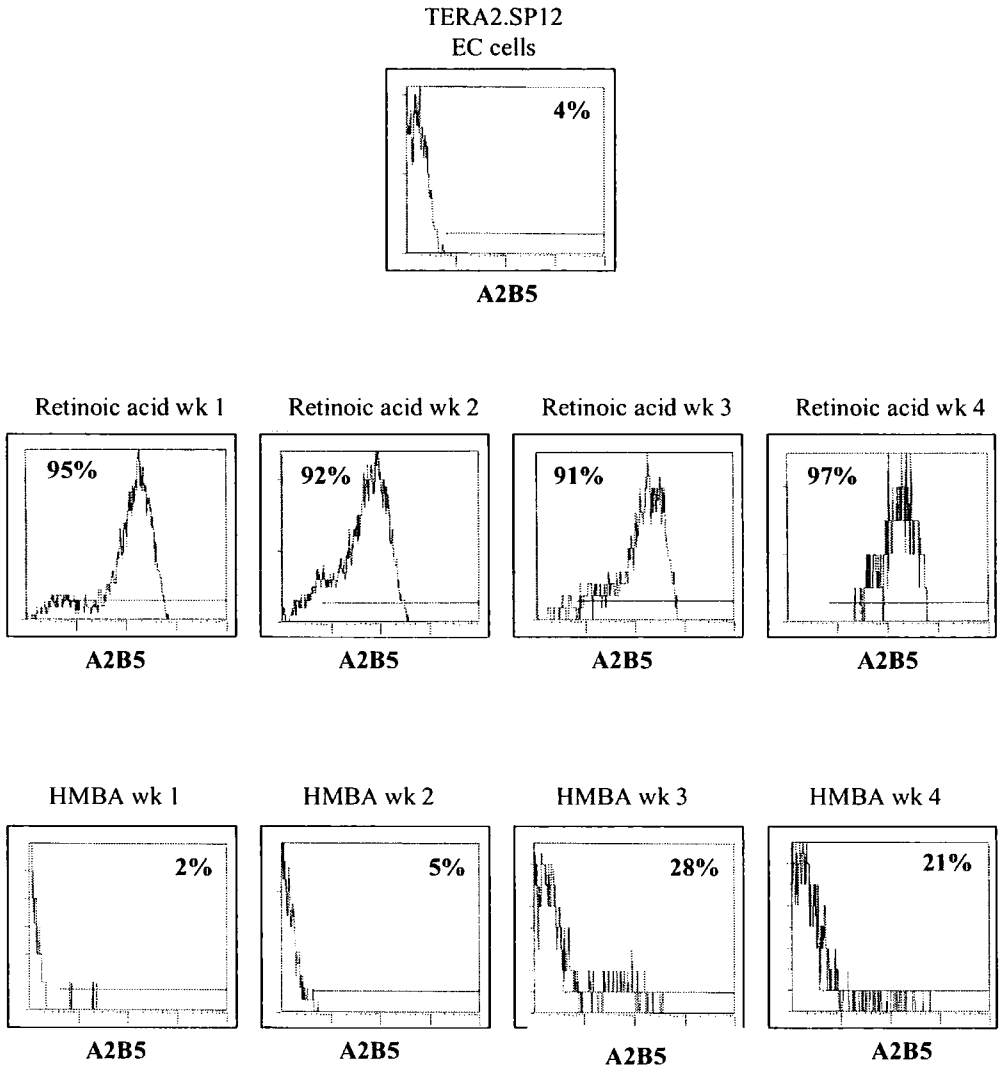


Figure 3.7: *Traces showing the change in expression of the differentiation marker VINIS-56 after differentiation of TERA2.SP12 EC cells with either retinoic acid (10μM) or HMBA (3mM) over a period of 4 weeks. Percentage value indicates the proportion of cells positively expressing VINIS-56. (x-axis=Intensity of cells expressing VINIS-56, y-axis=number of cells flourescing)*

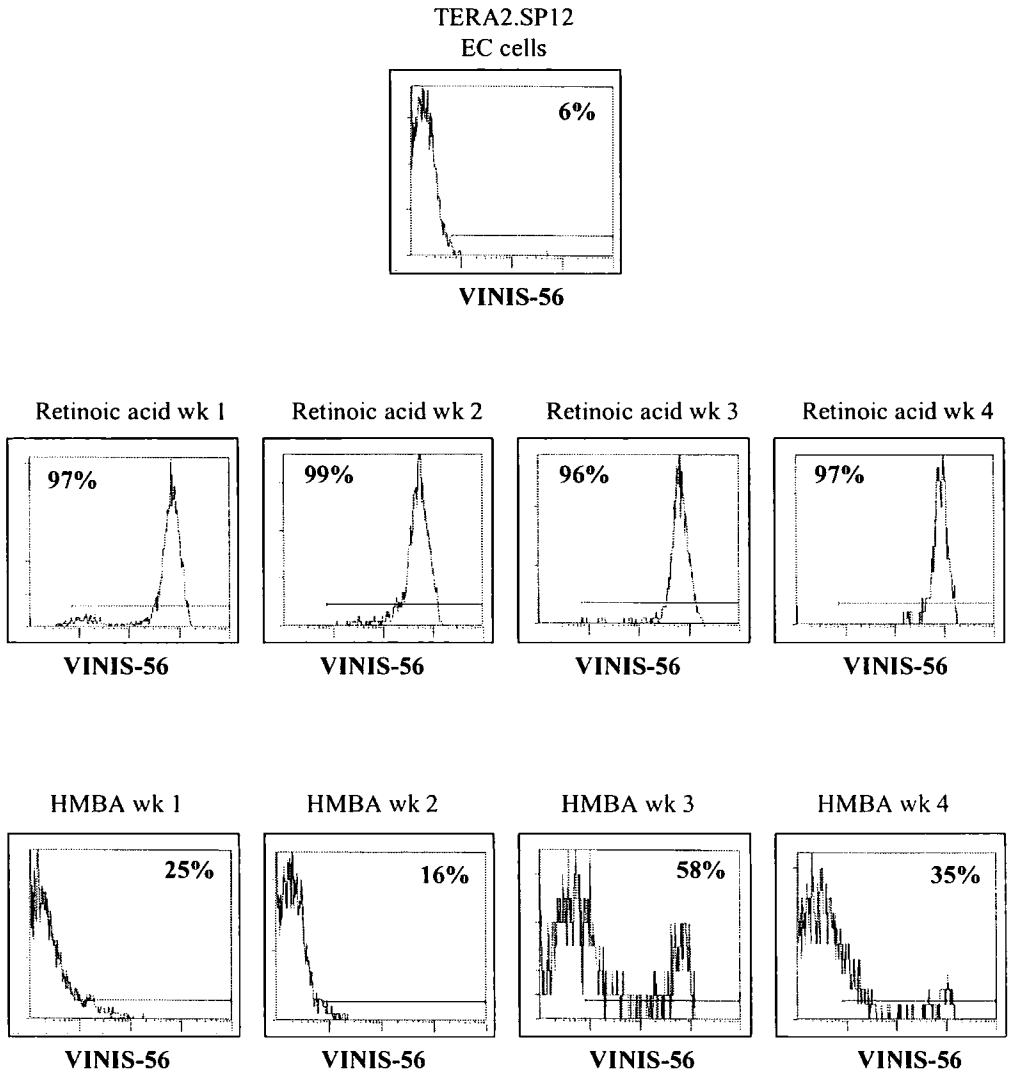


Figure 3.8: *Traces showing the change in expression of the differentiation marker Me311 after differentiation of TERA2.SP12 EC cells with either retinoic acid (10μM) or HMBA (3mM) over a period of 4 weeks. Percentage value indicates the proportion of cells positively expressing Me311. (x-axis=Intensity of cells expressing Me311, y-axis=number of cells flourescing)*

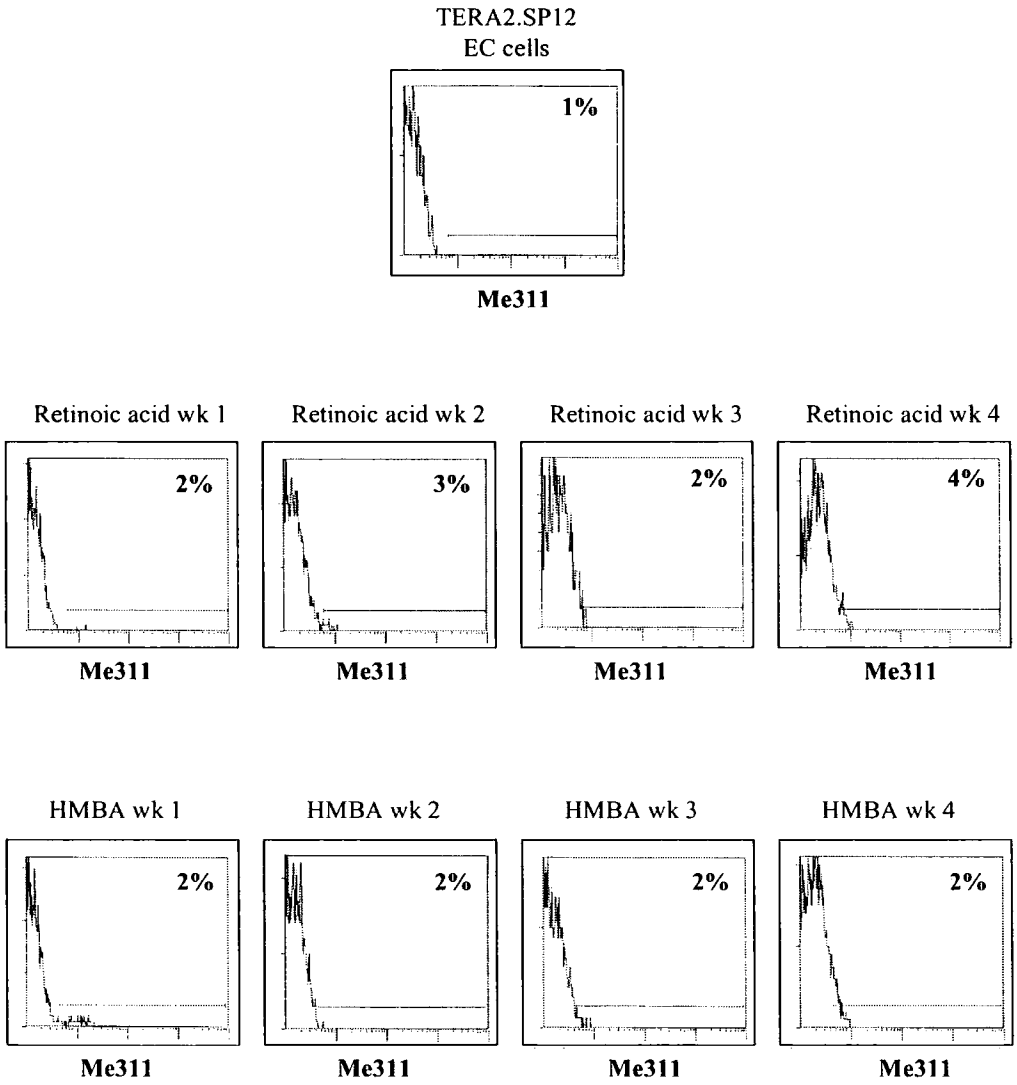
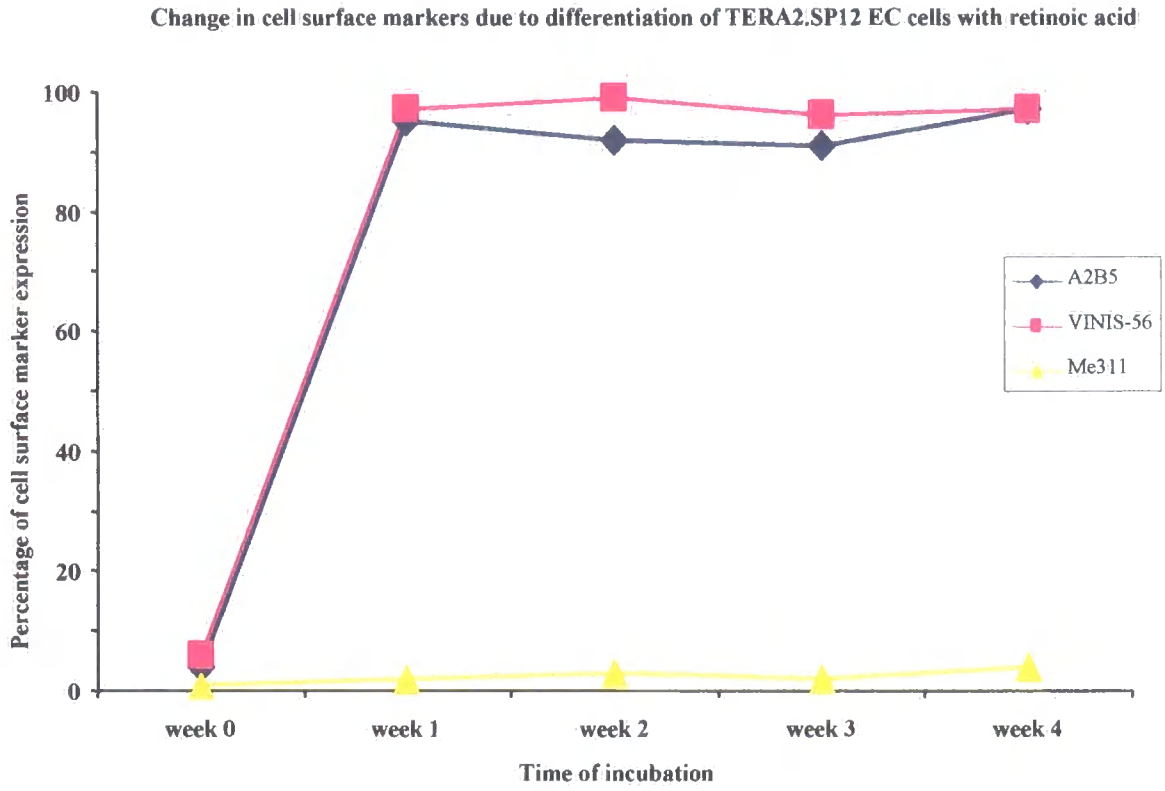
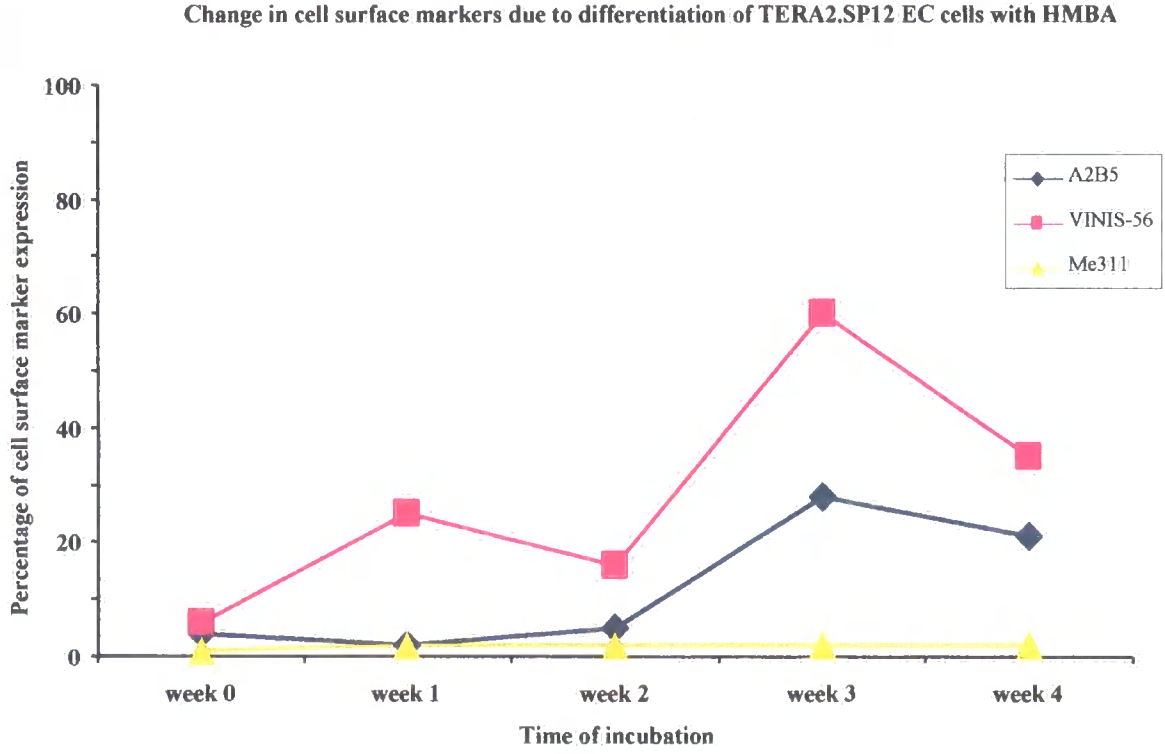


FIGURE 3.9: ***Overview of the expression of the differentiation markers during cellular differentiation.*** *TERA2.SP12 EC cells were exposed to either a) retinoic acid (10μM) or b) HMBA (3mM). Data were determined by flow cytometry. These figures represent typical data trends seen in all 3 separate data sets.*

a)



b)



3.3 Protein Expression Profiling during Embryonal Carcinoma Differentiation:

Protein expression profiles of specific markers for neural and non-neural cell types, including epidermis, were determined using western blot analysis during the differentiation of human EC cells (Figure 3.10). Proteins known to be expressed in neural cell types were detected by antibodies specific for NSE and NF-68, and antibodies against non-neural proteins such as keratin, cytokeratin-8 were used in this instance as there band sizes were easily visualised on a 10% acrylamide gel. NSE was expressed to a much higher level with retinoic acid induced cultures than with HMBA induced cultures. In both cases the level of expression increased in relation to the number of weeks the cultures had been exposed to the differentiation-inducing agents. NF-68 also showed increased expression in response to retinoic acid, but no NF68 expression was detected in HMBA treated cultures. Markers of epidermal derivatives including keratin and cytokeratin-8 were also studied. A general marker of keratin was broadly reactive to all samples tested, showing a slight increase in expression levels in cultures exposed to HMBA. In contrast, cytokeratin-8, a more specific marker of epidermal keratin was differentially expressed in the treated samples. Cytokeratin-8 was expressed at very low levels in the EC cells and showed a marked increased of expression in samples of differentiated cells. HMBA- treated cells showed significantly higher levels of cytokeratin-8 expression compared to those induced to differentiate by retinoic acid.

Graphs were plotted of densitometry data obtained from studying individual band density using pixels (Figure 3.11)

FIGURE 3.10: ***Profiles of protein expression were determined by western analysis during the differentiation of TERA2.SP12 EC cells in response to retinoic acid (RA) and HMBA.*** *Markers for neural cell types were detected by antibodies specific for NSE and NF-68, whilst epidermal and extracellular matrix proteins were detected by keratin and cytokeratin-8. This analyses was preformed on 2 data sets, both of which produced similar results. β-actin was used as a loading control.*

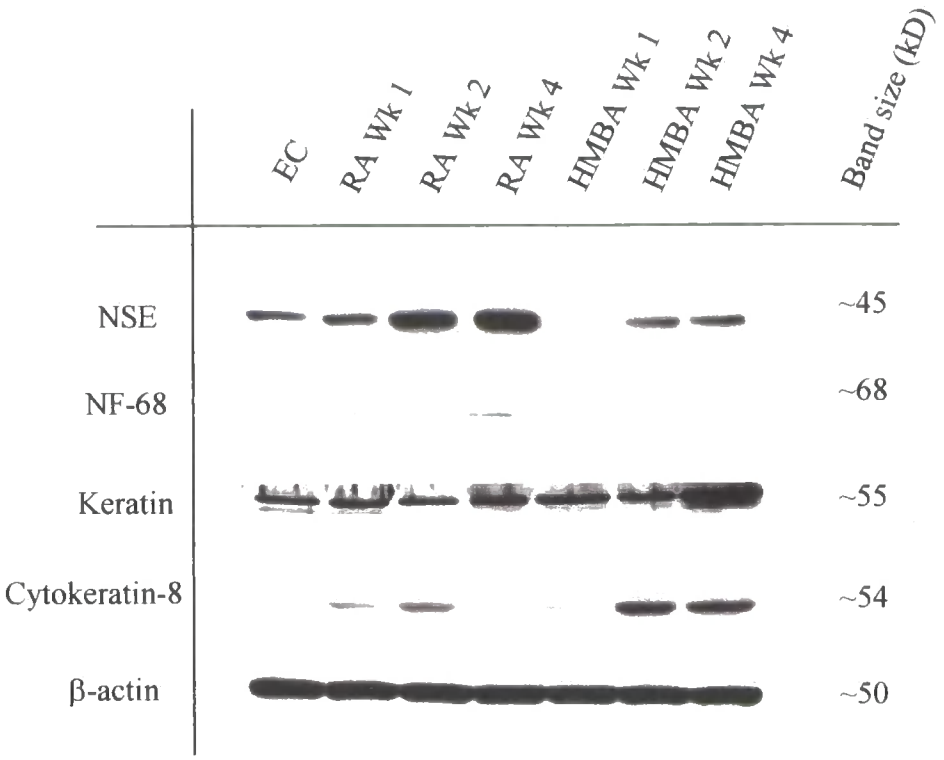
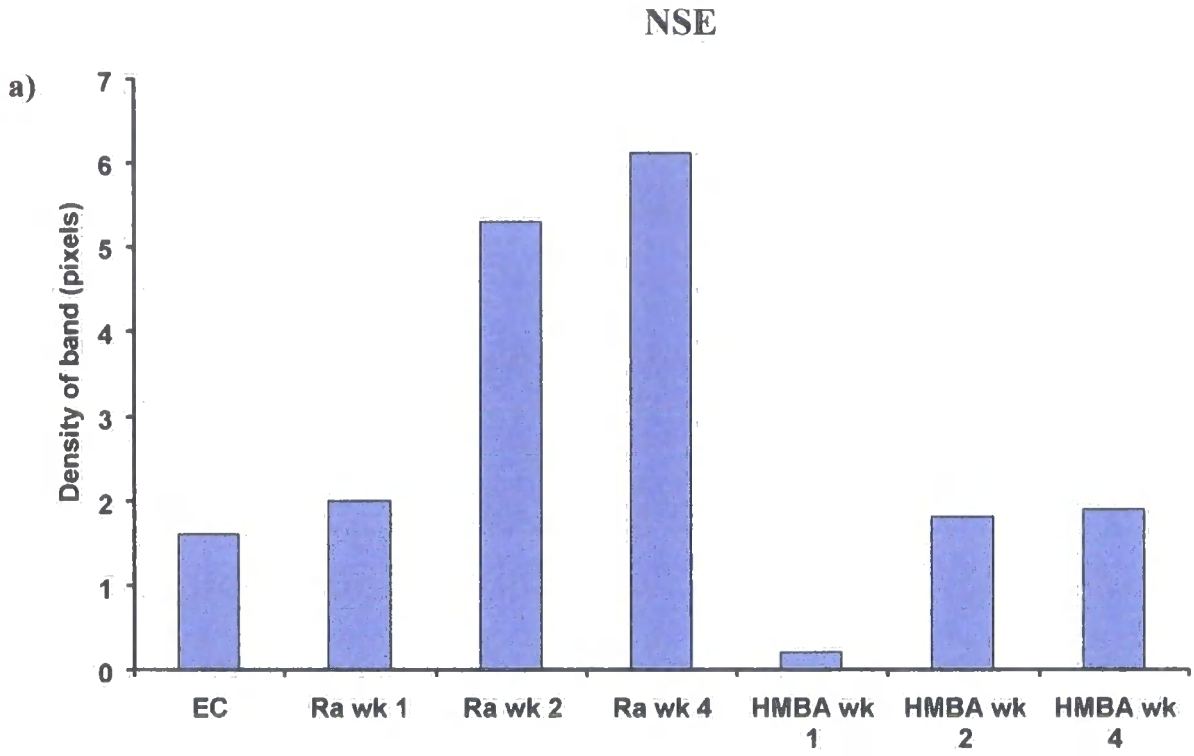


FIGURE 3.11: ***Densitometric analysis of banding pattern from western blots showing profiles of protein expression during differentiation of TERA2.SP12 EC cells.*** *Graphs show plots for a) NSE b) NF-68 c) Keratin d) Cytokeratin-8 and e) β-actin. This analyses was preformed on 2 data sets, both of which produced similar results.*



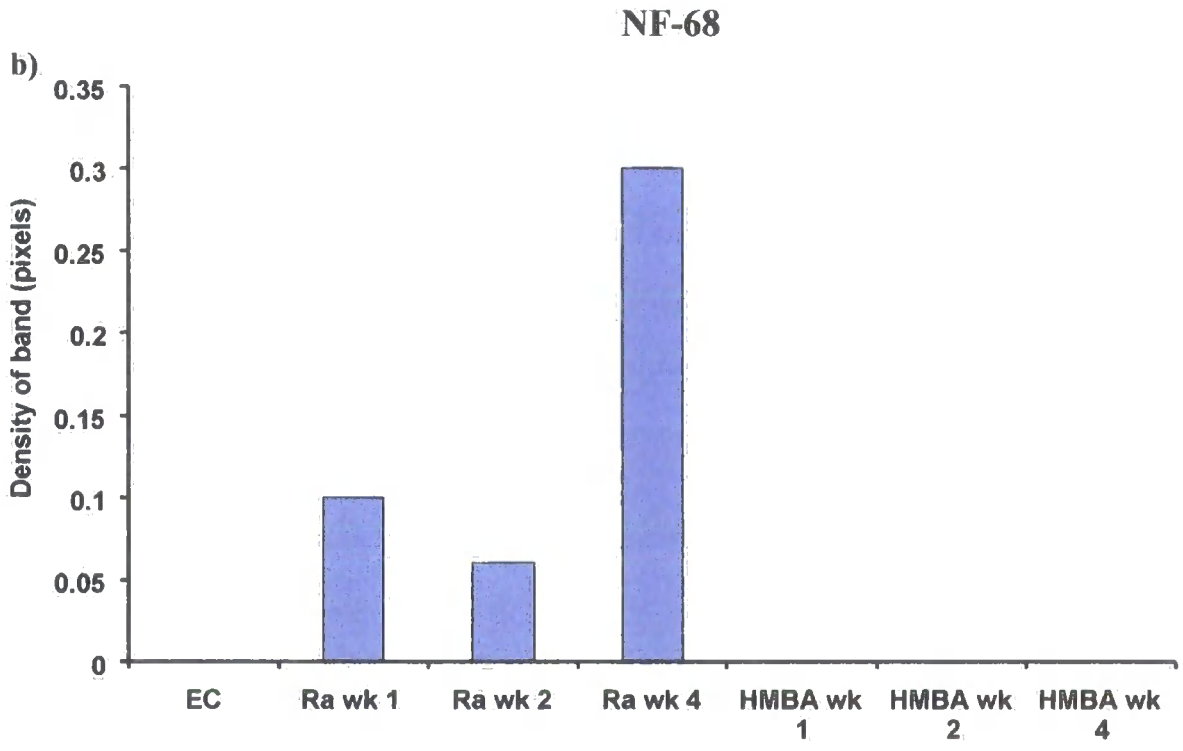
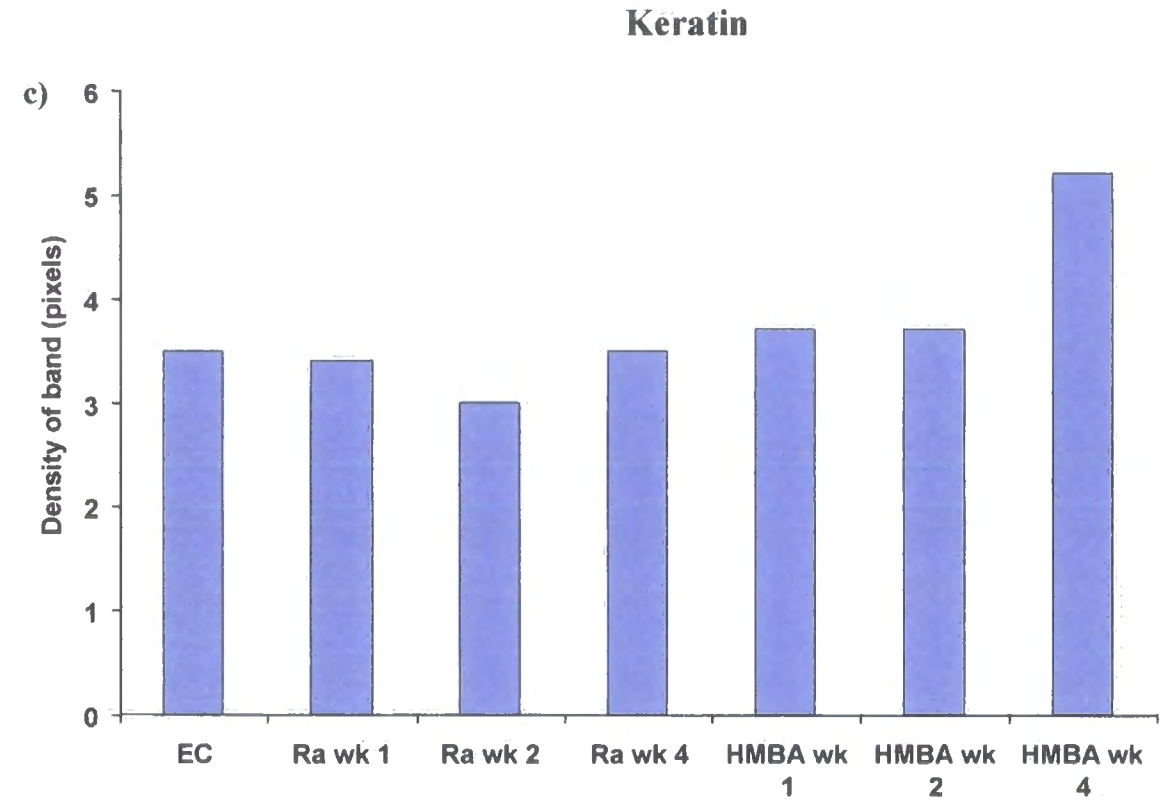


FIGURE 3.11: *Continued…*



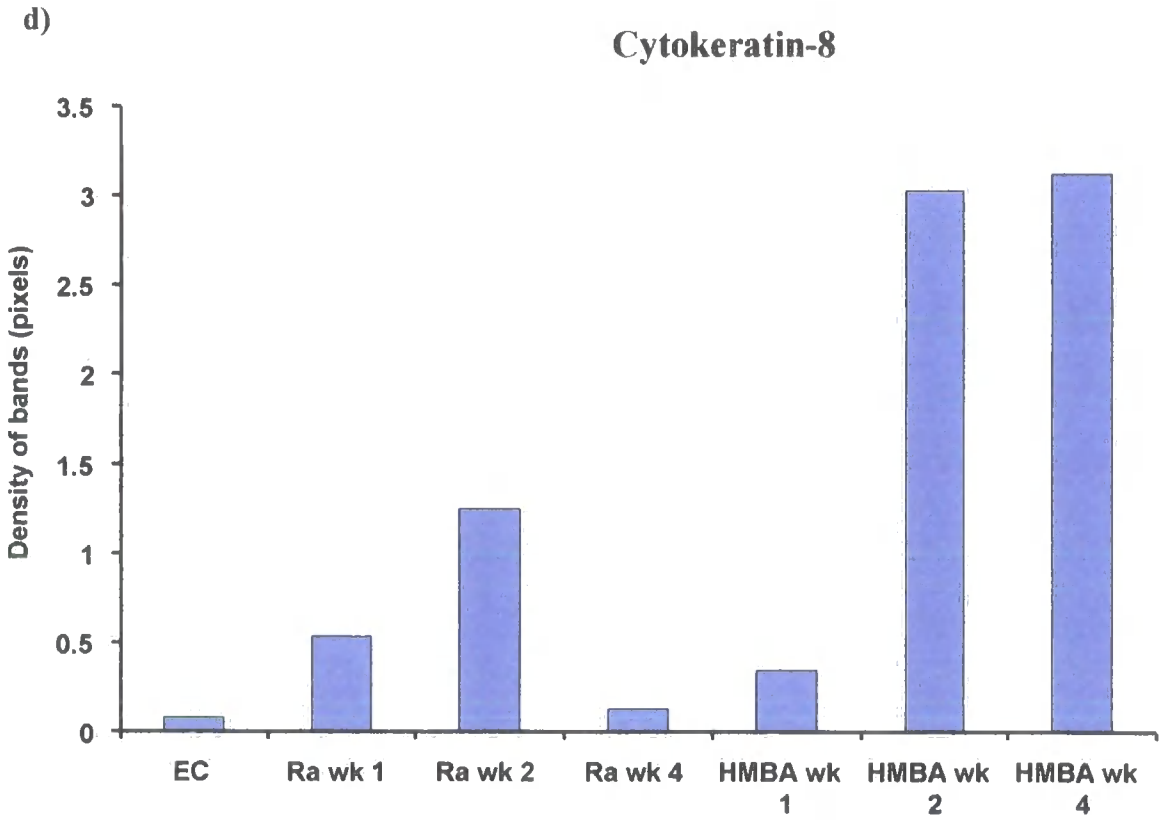
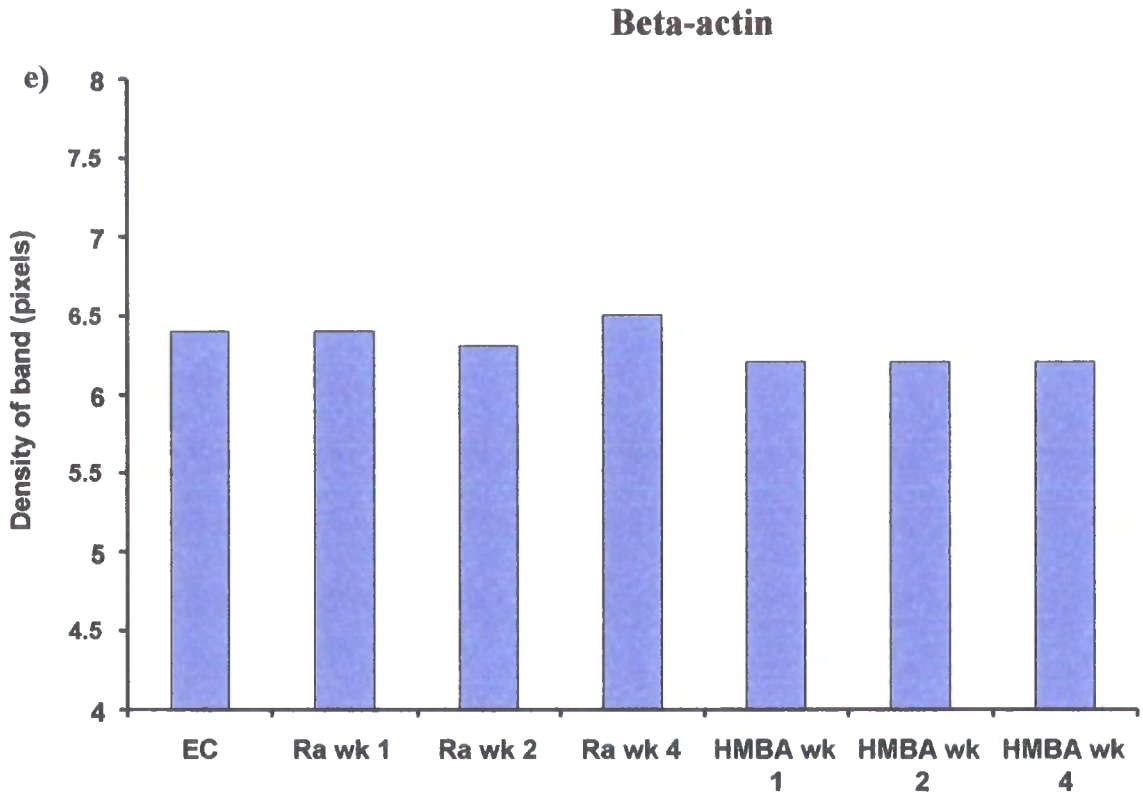


FIGURE 3.11: ***Continued…***



3.4 Immunofluorescence Localisation of Protein Markers during Embryonal Carcinoma Cell Differentiation:

To back up the data produced from the western blot profiles more markers of neural and non-neural/epithelial cell types were analysed. More non-neural/epithelial cell markers were investigated, as looking at the morphology and western data produced it is possible that some of the non-neural cell types seen could be of a epithelial nature. The markers of neural proteins used were β-tubulin-III and NF-68, and markers of non-neural/epithelial proteins were cytokeratin-8, fibronectin and laminin. Using immunofluorescent techniques neurons were identified by the marker β-tubulin-III, which was found to be expressed in distinct islands of neuronal cells in cultures induced to differentiate with retinoic acid. It was also possible to see neurites around the edge of the islands expressing β-tubulin-III. However, β-tubulin-III expression was completely absent in HMBA-induced differentiated cells (Figure 3.12). Cytokeratin-8, an epidermal associated marker, was expressed in significantly higher levels in samples induced to differentiate with HMBA than those induced to differentiate with retinoic acid (Figure 3.13). This observation is in agreement with the western analysis data. The fibronectin antibody recognises an extra-cellular protein associated with the extra-cellular matrix. It has been associated with epidermal layer development (Grover et al 1983), however, it is not an exclusive epidermal marker, as it also stains mesenchyme. This factor has to be considered when interpreting the results obtained. In TERA2.SP12 cultures the expression of fibronectin also appears to be significantly increased in the cultures induced to differentiate with HMBA compared with the level seen in cells induced to differentiate with retinoic acid (Figure 3.14). It is also noted that there are differences in cell density between the figures shown in figure 3.14, this may have some affect on the level of fibronectin expression, as it is generally found to be up-regulated with increased density. Anti-laminin

shows immunoreactivity to laminin found in basement membranes. As well as fibronectin, laminin is associated with the formation of epithelial layers during development (Grover et al 1983). Again, HMBA treated cultures showed higher levels of laminin expression. Large thick tracks of laminin protein were visible compared to a fine network observed in retinoic acid treated cultures (Figure 3.15).

TERA2.SP12 EC cells were also grown and induced to differentiate as aggregates. We have previously shown that when aggregates are kept in suspension and exposed to retinoic acid, roughly 95% of cells within the aggregate take on a neuronal phenotype (Stewart et al 2003). HMBA aggregates have not previously been studied in this particular cell line. In this study aggregates induced to differentiate with either retinoic acid or HMBA were grown in suspension for 3 weeks followed by a further 3 weeks growth on coverslips. Aggregate formations were visualised in both cultures. Cells were stained for specific proteins indicative of certain cell types. Entire aggregates stained positive for β-tubulin-III in the retinoic acid induced samples. Some low level β-tubulin-III staining was picked up in the HMBA induced samples (Figure 3.16). High levels of cytokeratin-8 staining was detected in response to retinoic acid and HMBA but especially throughout retinoic acid-induced cultures (Figure 3.17). In addition, cells that had appeared to have spread away from the retinoic acid-induced aggregates showed intricate staining of cytokeratin-8 (Figure 3.17e). Retinoic acid-induced aggregates all stained positive for NF-68 whereas cells which had spread away from the main body of the aggregate were negative, suggesting that no new neuronal growth away from the aggregate was occurring (Figure 3.18). Very few cells stained positive for NF-68 in cultures grown as aggregates in the presence of HMBA indicating little neuronal growth.

FIGURE 3.12: ***Immunocytochemical staining with β-tubulin-III of TERA2.SP12 EC cells after 4 weeks differentiation using either retinoic acid (a,b) or HMBA (c,d).*** *Phase contrast image of retinoic acid induced differentiated EC cells (a) and the corresponding fluorescent image (b). Phase contrast image of HMBA-induced differentiated EC cells (c) and the corresponding fluorescent image (d). Note the presence of distinct islands of cells that express β-tubulin-III in retinoic acid induced differentiated cells (arrows on phase photograph) and the presence of neurites (arrow on fluorescent image). Few similar such structures and almost no β-tubulin-III expression was observed in the HMBA induced cultures. Bars: 400μm*

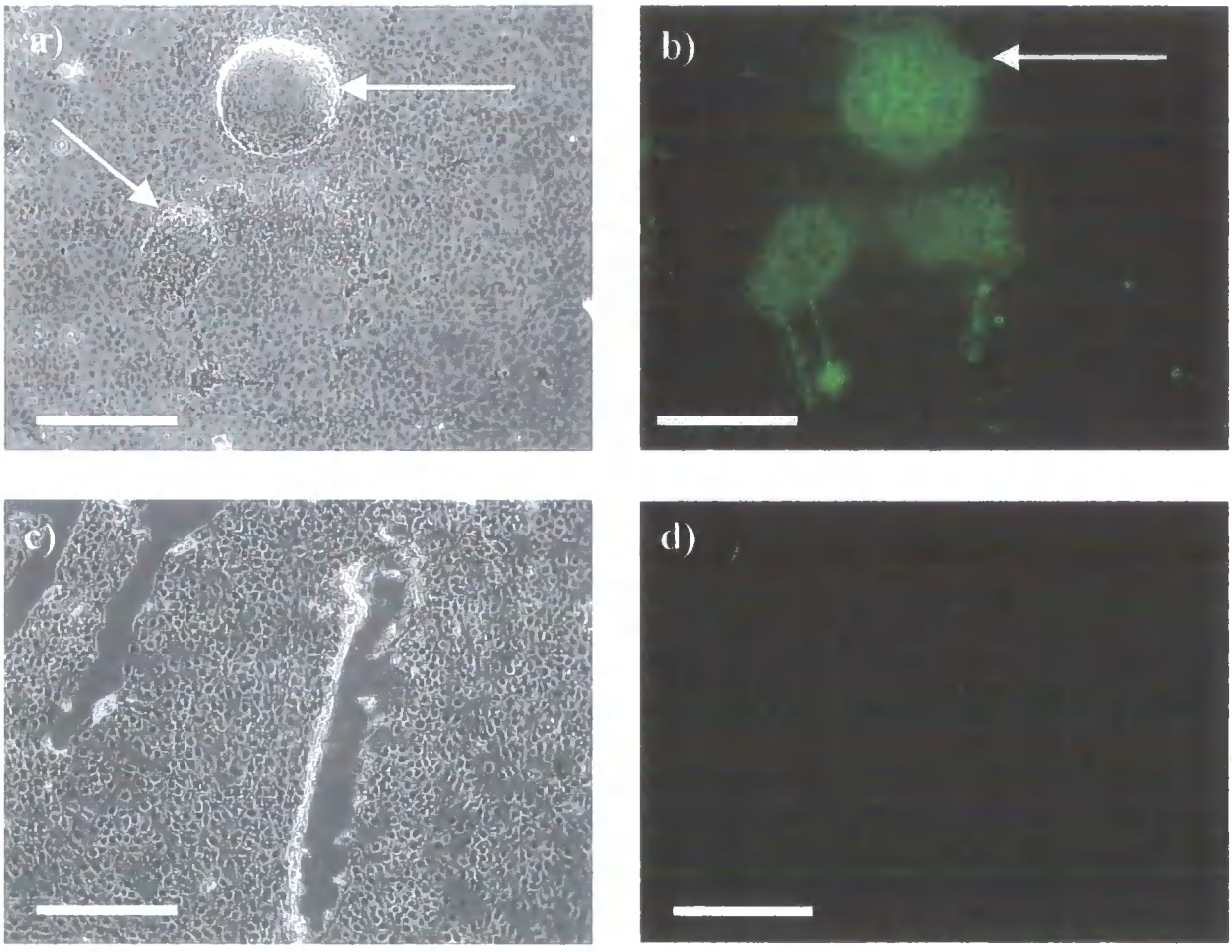


FIGURE 3.13: ***Immunocytochemical staining using cytokeratin-8 on TERA2.SP12 EC cells after 4 weeks differentiation using either retinoic acid (a,b) or HMBA (c,d).*** *Phase contrast image of retinoic acid induced differentiated EC cells (a) and (b) corresponding fluorescent micrograph. Images show a typical region of non-neuronal cells induced by retinoic acid. Phase contrast image of HMBA-induced differentiated EC cells (c) and (d) corresponding fluorescent image. Note the higher expression level of cytokeratin-8, a marker of epidermal cells, in HMBA induced differentiated cells. This pattern was typical across the culture. Bar: 150μm*

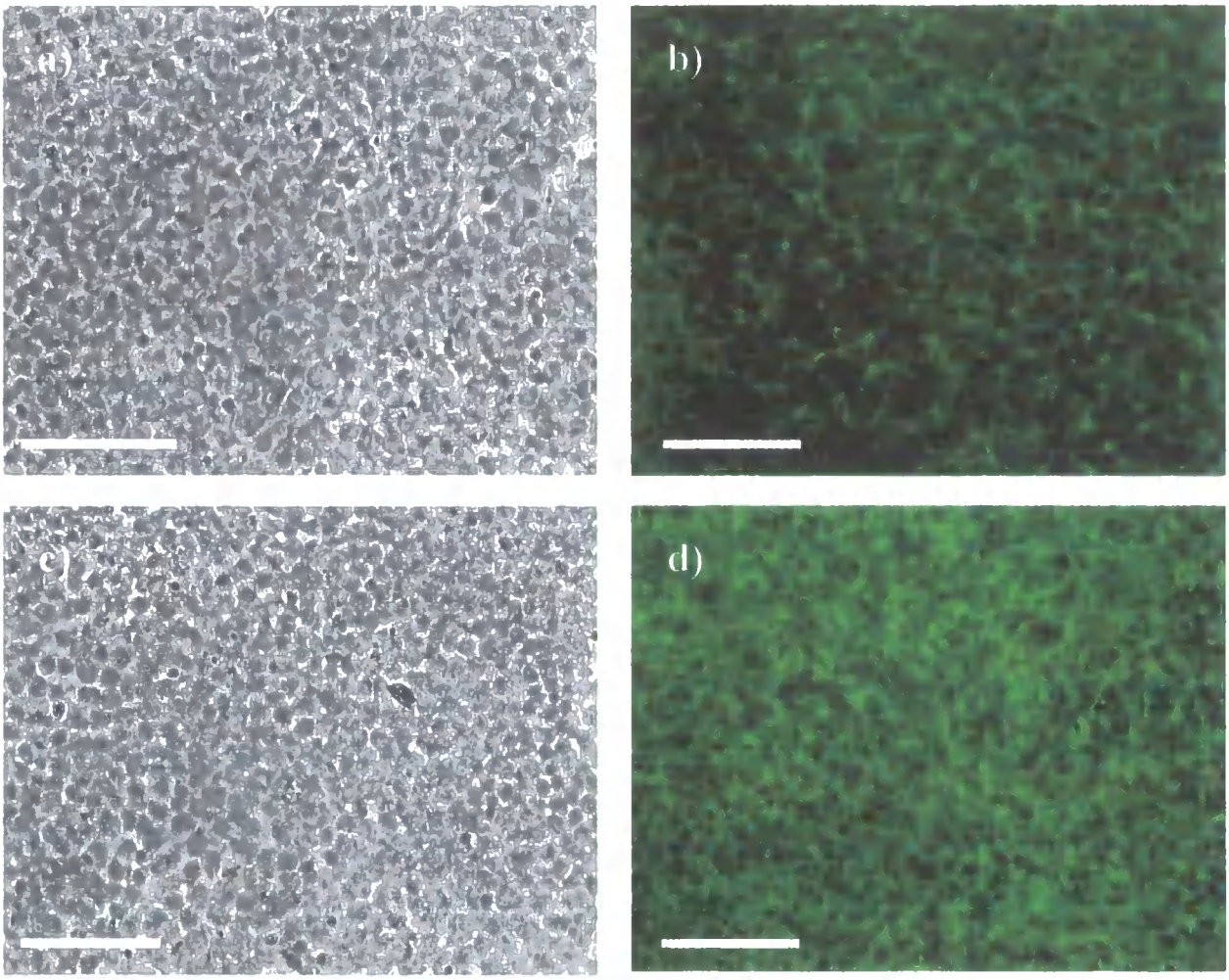


FIGURE 3.14: ***Immunocytochemical staining using fibronectin on TERA2.SP12 EC cells after 4 weeks differentiation using either retinoic acid (a,b) or HMBA (c,d).*** *Phase contrast image of retinoic acid induced differentiated EC cells (a) and (b) corresponding fluorescent image. Phase contrast image of HMBA-induced differentiated EC cells (c) and (d) corresponding fluorescent image. Note the increased intensity and quantity of staining in response to HMBA differentiation. This pattern was typical across the culture. Bar: 250μm*

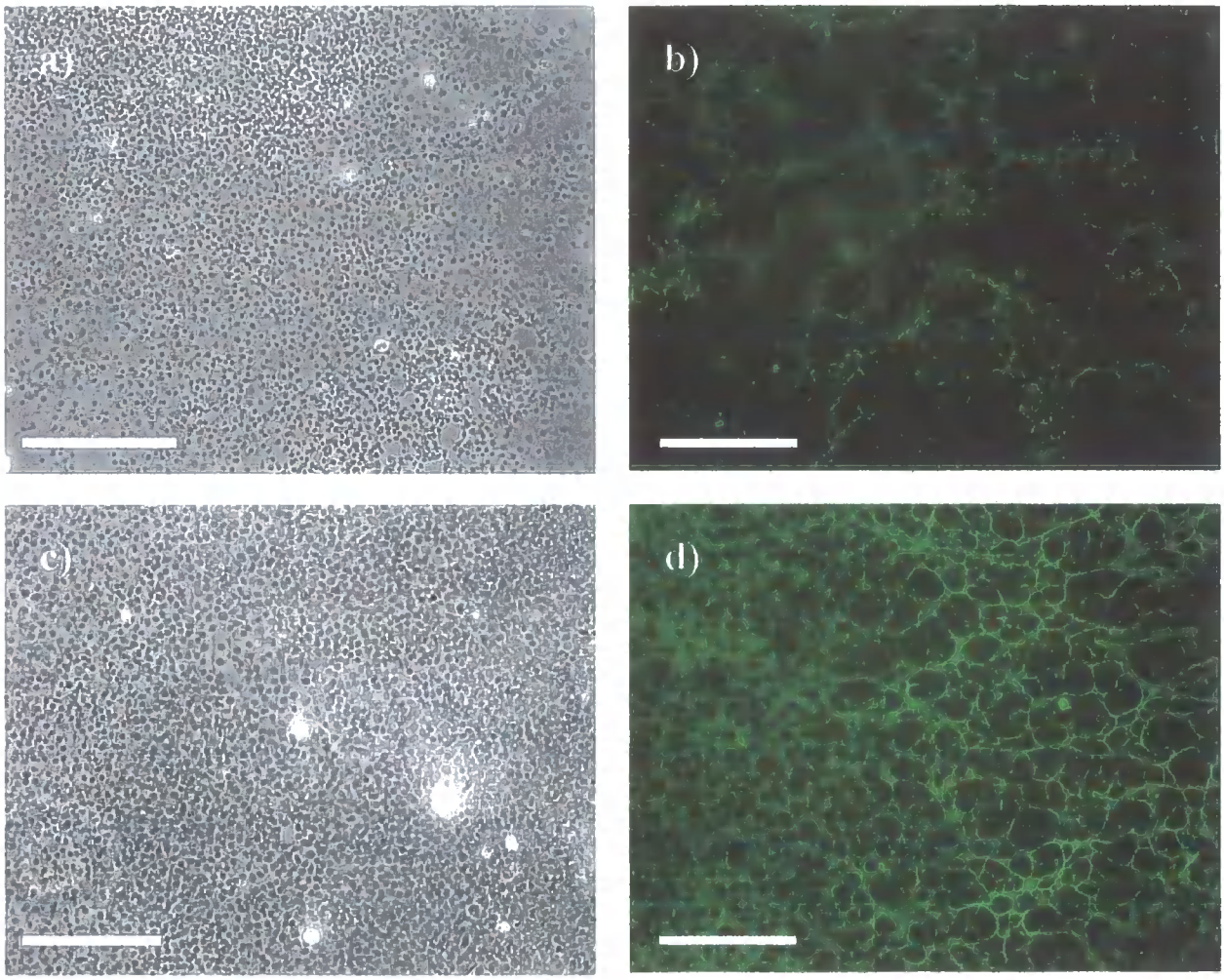
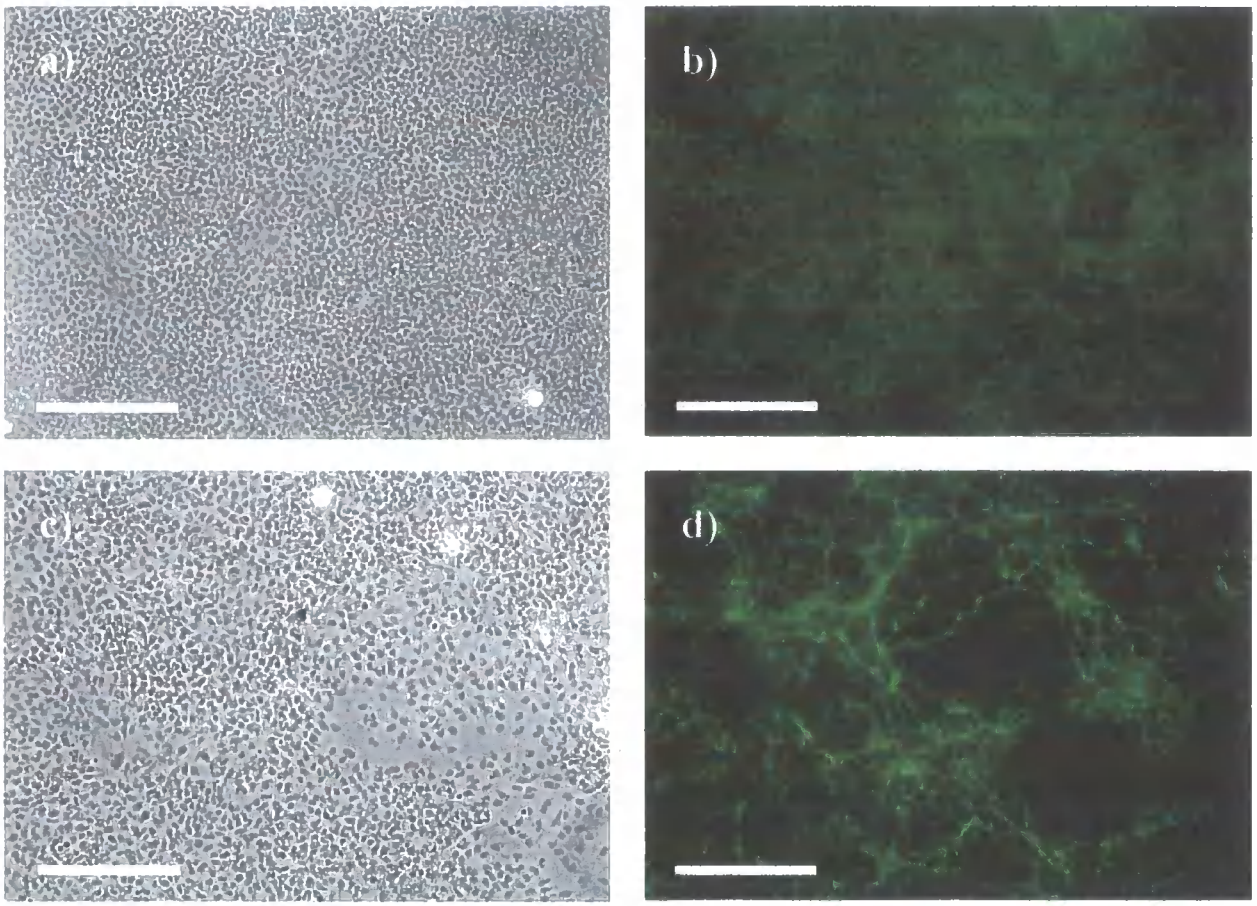


FIGURE 3.15: ***Immunocytochemical staining with laminin of TERA2.SP12 EC cells after 4 weeks differentiation using either retinoic acid (a,b) or HMBA (c,d).*** *Phase contrast image of retinoic acid induced differentiated EC cells (a) and (b) corresponding fluorescent image. Phase contrast image of HMBA-induced differentiated EC cells (c) and (d) corresponding fluorescent image. Note the different expression patterns in the different cultures: Large amounts of laminin arranged in thick bands are seen in HMBA-induced cultures compared to a fine network of staining seen around the cells in retinoic acid induced cultures. This pattern was consistent across the culture. Bar: 250μm*



To examine the degree of epidermal differentiation, keratin-14 was used to detect more mature epidermal structures. Under these culture conditions, staining for keratin-14 was negative in all samples of TERA2.SP12 cultures tested (Figure 3.19). A positive control sample of skin was also tested, the basement membrane and hair follicles showed high expression of keratin-14 indicating positive staining for the antibody (Figure 3.19e and f).

When TERA2.SP12 EC cells were exposed to either retinoic acid or HMBA either as monolayer cultures or grown as aggregates, it was noted that cells which stained for markers of neural lineages, such as β-tubulin-III and NF-68 did not stain positively for the non-neural markers fibronectin, cytokeratin-8 or laminin. This is best seen in figures 3.16 and 3.18, where the larger, flatter-looking cells do not express the neural markers, whereas cells which are of a similar nature shown in figure 3.17 are positive for cytokeratin-8.

FIGURE 3.16: ***Immunocytochemical staining with β-tubulin-III of differentiated TERA2.SP12 EC cells grown as aggregates.*** *Cells were initially grown as aggregates for 3 weeks followed by 3 weeks growth on glass coverslips. Phase contrast image of retinoic acid induced (10μM) differentiated cells (a) and (b) corresponding fluorescent image. Phase contrast image of HMBA (3mM) induced differentiated cells (c) and (d) corresponding fluorescent image. Low power magnification image of (e) retinoic acid-induced cells and (f) HMBA-induced cells. Large numbers of neurons were observed in retinoic acid induced cultures whilst few were noted in cultures exposed to HMBA. The arrow indicates a possible neurite present in the HMBA aggregate cultures (arrows in c and d). Bar: (a,b,c,d) 100μm and (e,f) 200 μm*

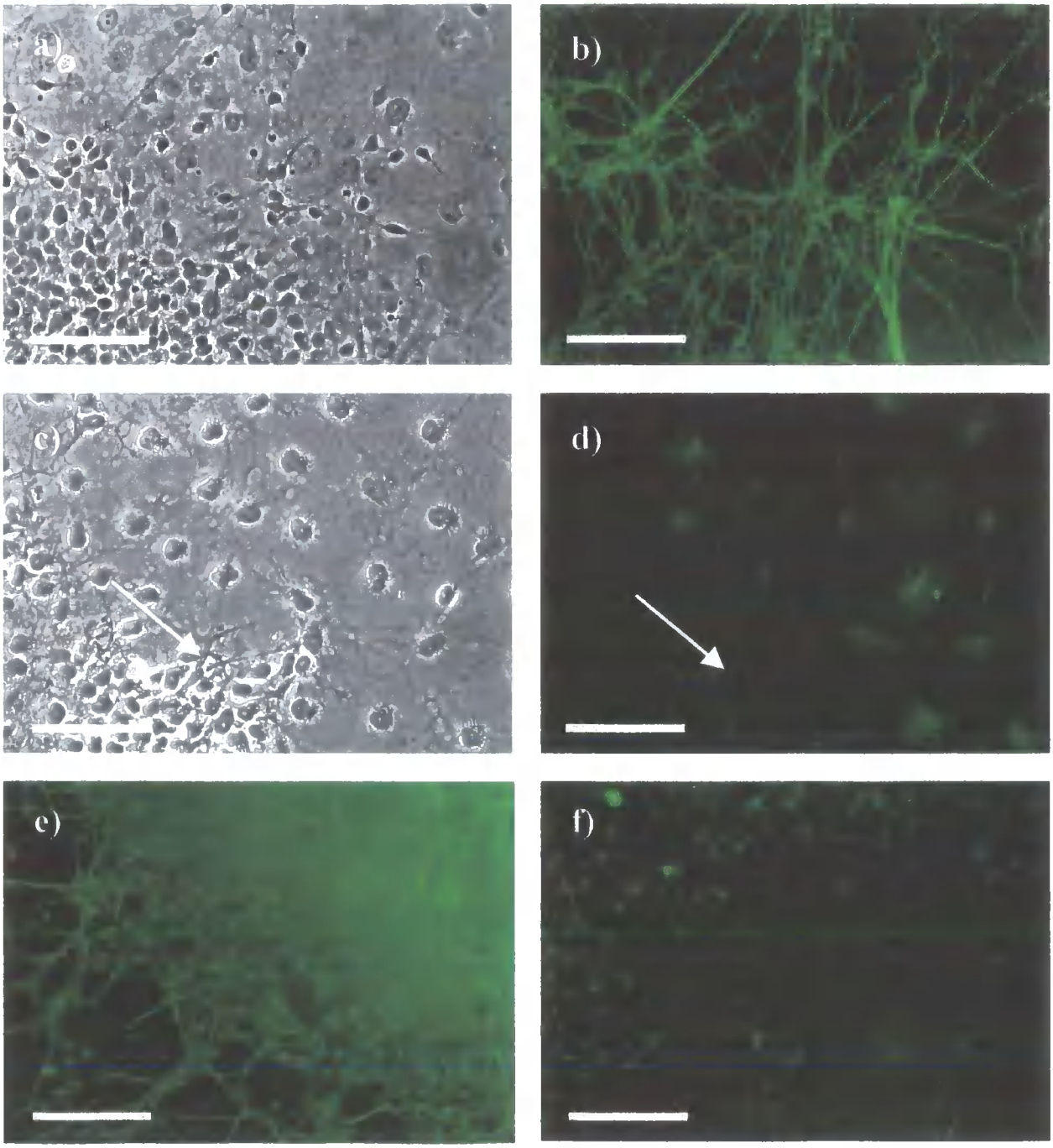


FIGURE 3.17: ***Immunocytochemical staining with cytokeratin-8 of differentiated EC cells grown as aggregates.*** *Cells were initially grown as aggregates for 3 weeks followed by 3 weeks growth on glass coverslips. Phase contrast images of retinoic acid induced (10μM) differentiated cells (a,c) and corresponding fluorescent image (b,d). Phase contrast image of HMBA (3mM) induced differentiated cells (e) and corresponding fluorescent image (f). Fluorescent image (d) shows cells remote from the aggregate that have spread out forming large flat structures expressing cytokeratin-8. Note the presence of neurons expressing neurites in the HMBA aggregate cultures (arrow). Bar: (a,b,e,f) 100μm and (c,d) 125μm*

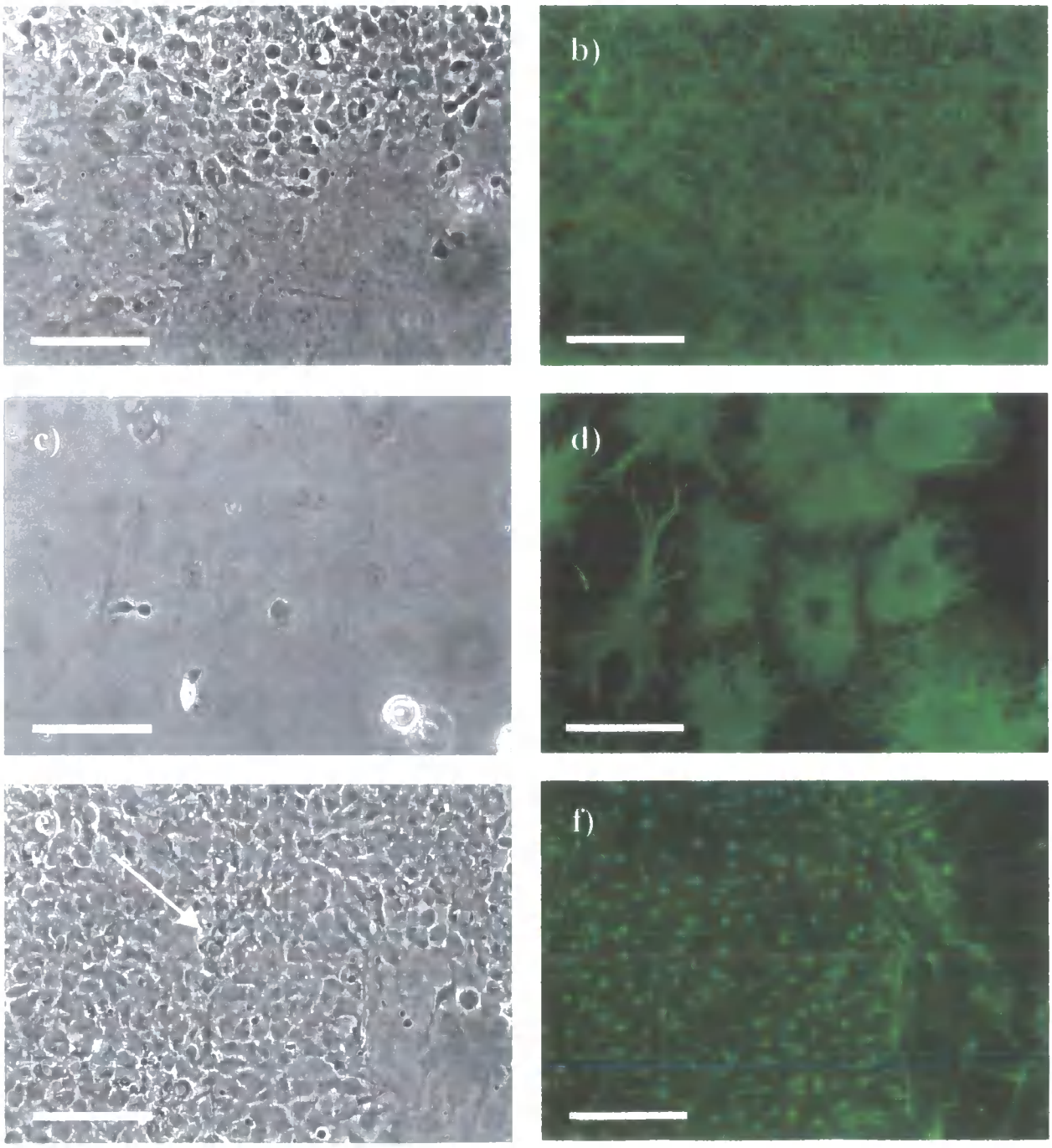


FIGURE 3.18: ***Immunocytochemical staining with NF-68 of differentiated TERA2.SP12 EC cells grown as aggregates.*** *Cells were initially grown as aggregates for 3 weeks followed by 3 weeks growth on glass coverslips. Phase contrast image of retinoic acid induced (10μM) differentiated cells (a) and corresponding fluorescent image (b). Phase contrast image of HMBA (3mM) induced differentiated cells (c) and corresponding fluorescent image (d). Note that the expression of NF-68 (a neural marker) is absent in the HMBA aggregates and expressed strongly in the retinoic acid cultures but only in the aggregate itself and not in the large flat cells away from the aggregate (arrow). Bar: 100μm*

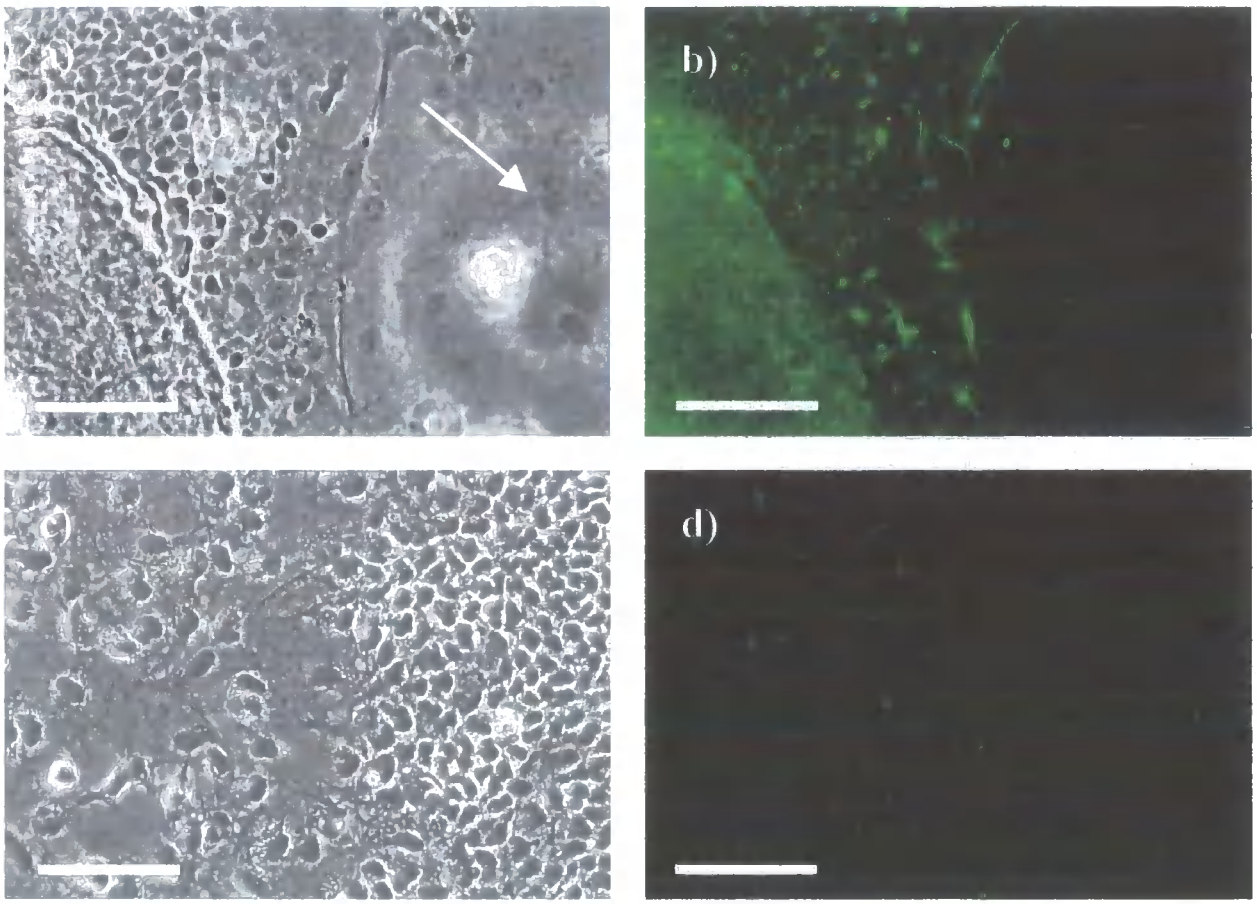
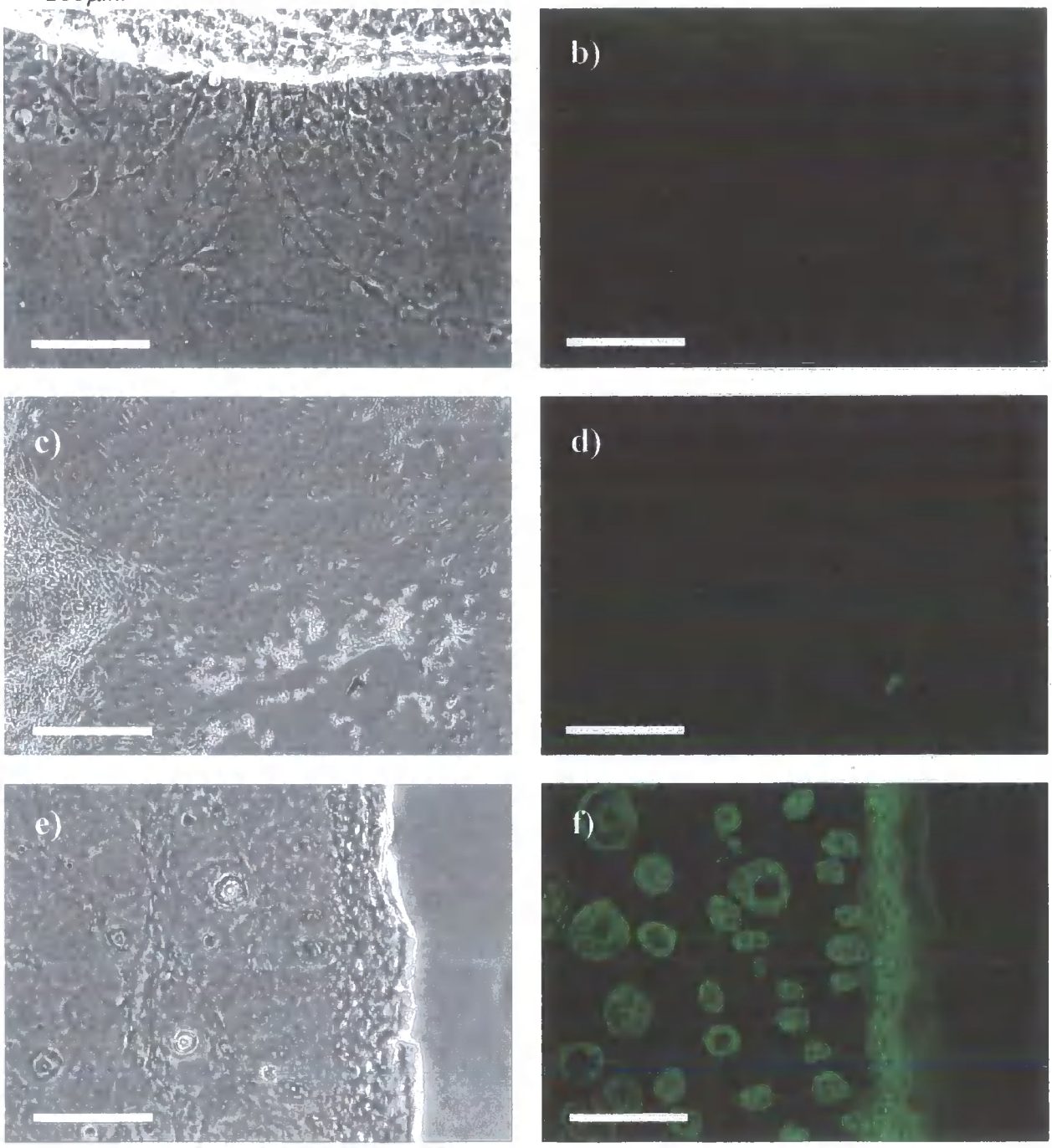


FIGURE 3.19: ***Immunocytochemical staining with keratin-14 (a mature epidermal marker) of differentiated TERA2.SP12 EC cells grown as aggregates.*** *Cells were initially grown as aggregates for 3 weeks followed by 3 weeks growth on glass coverslips. Phase contrast image of retinoic acid induced (10μM) differentiated cells (a) and corresponding fluorescent image (b). Phase contrast image of HMBA (3mM) induced differentiated cells (c) and corresponding fluorescent image (d). Control phase contrast image of a sectioned mammalian skin sample (e) and corresponding fluorescent image (f) (sample courtesy of Dr A Maatta). No significant expression of keratin-14 can be seen with either of the differentiated EC cell samples suggesting no mature epidermis being present. In contrast, strong expression of the antibody was seen associated with the suprabasal layers and hair follicles within the skin. Bar: (a,b,e,f) 100μm, (c,d) 200μm.*



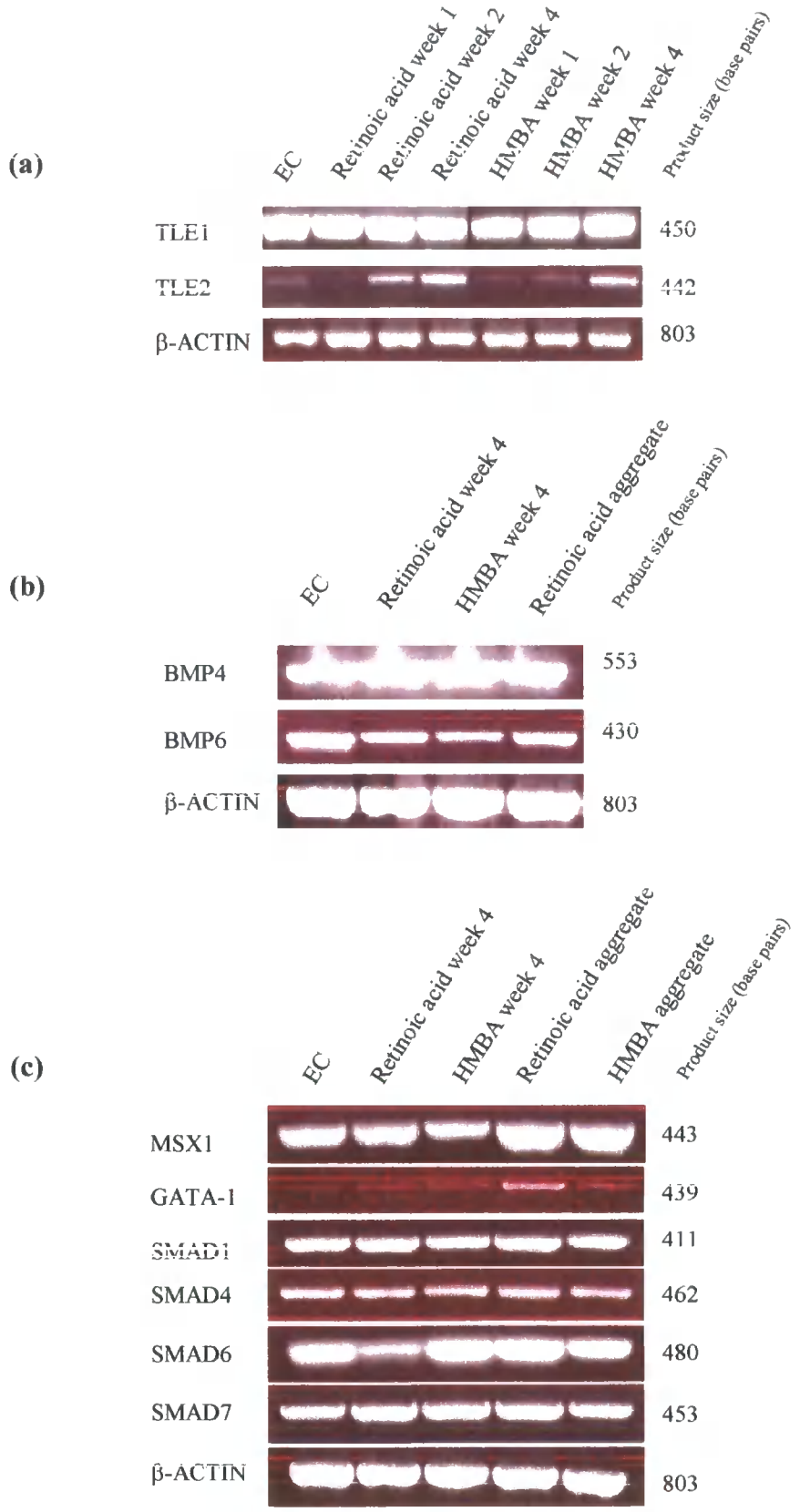
3.5 Transcriptional Profiling of Differentiating Embryonal Carcinoma Cells:

RNA was extracted from cultures of TERA2.SP12 stem cells and their differentiated derivatives and used to analyse the expression profiles produced of specific anti-neural genes and molecules involved in the bone morphogenetic protein signalling pathway by RT-PCR.

Anti-neural genes were examined to see if the absence of neural cells within HMBA-induced cultures occurred as a result of the activation of specific anti-neural pathways. The anti-neural gene *tle1* was expressed evenly throughout all the samples, whereas *tle2* was expressed in increasing intensity as the incubation period of the cultures with retinoic acid and HMBA increased (Figure 3.20a).

As the differentiation patterns seen with TERA2.SP12 EC cells appear to represent those seen in the ectoderm of the developing human embryo, expression of specific regulatory proteins from this process were studied, namely those associated with the bone morphogenetic protein signalling pathway (reviewed in Weinstein and Hemmati-Brivanlou 1999). Bone morphogenetic proteins (BMP) 4, and 6 were expressed in all samples tested (Figure 3.20b). Molecules which are found further down the BMP signalling pathway including, *msx1*, *Smad1,4,6* and *7*, also showed similar levels of expression under the different culture conditions. Interestingly, *gata-1* expression was highly regulated, showing highest levels of transcription in cultures grown initially as aggregates in response to retinoic acid (Figure 3.20c).

FIGURE 3.20: ***Transcriptional profiling of gene expression during TERA2.SP12 EC cell differentiation as determined by RT-PCR.*** *β-actin was used as a loading/positive control in each case. The size of each amplified product agreed with values as expected.*



3.6 Summary of Results

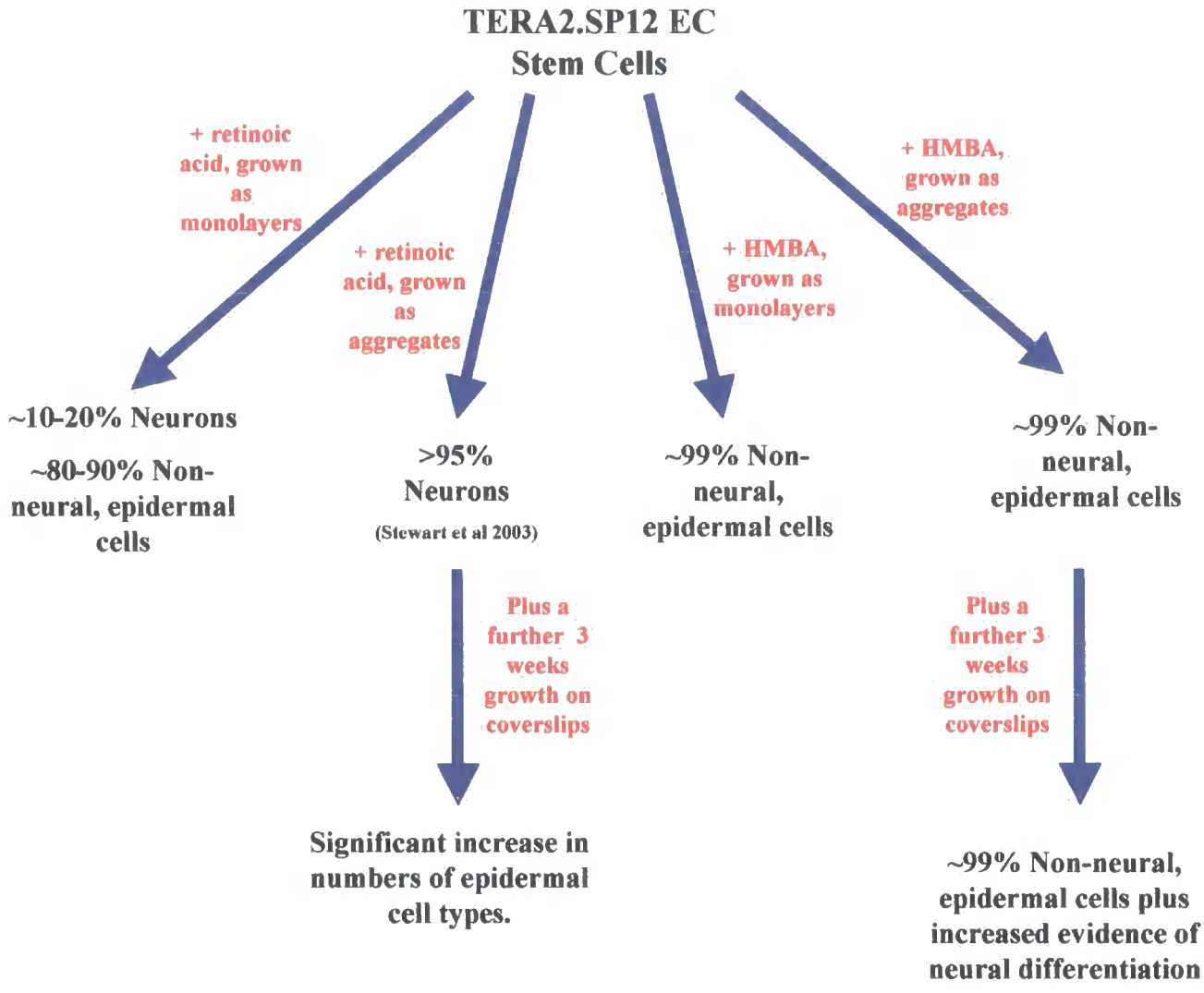
TERA2.SP12 EC stem cells can be induced to differentiate along different pathways by exposure to either retinoic acid, HMBA, or by altering the culture conditions. Cells cultured as monolayers with retinoic acid appear to produce ~10-20% neuronal type cells and ~80-90% non-neural cells. When the cells are grown as aggregates they produce >95% neuronal tissues when exposed to retinoic acid. However when these aggregates are grown on coverslips for prolonged periods, an increased level of non-neural cell types are seen.

When cells are exposed to HMBA and grown as aggregates ~95% of cells appear to take on a non-neural lineage, but a small percentage of cells also appear to take on a neural phenotype when the aggregates undergo extended growth on coverslips. In contrast, TERA2.SP12 cells grown as monolayers in the presence of HMBA take on a ~99% non-neuronal cell phenotype with neurons seldom being seen.

In all cultures tested this non-neuronal cell type appears to be epithelial in character.

A diagrammatical overview of the results obtained in this investigation can be seen in Figure 3.21.

FIGURE 3.21: ***General Overview of the Alternative Pathways of Cellular Differentiation during the Growth of Human TERA2.SP12 EC Stem Cells Under Varying Cell Culture Conditions.***



Chapter 4

Discussion

4.1 Neural and Non-neural Differentiation of TERA2.SP12 Embryonal Carcinoma Stem Cells

The ability of human EC cells to differentiate into neural sub-types both spontaneously and in response to retinoic acid has been well documented. For instance, Pera et al (1989) demonstrated the ability of the germ cell tumour (GCT) derived EC cell line GCT 27 to differentiate spontaneously into many tissue types including neuroectodermal cells. The controlled differentiation of human EC cell lines in response to retinoic acid has also been extensively studied (Andrews et al 1984; Przyborski et al. 2000; Thompson et al 1984; Simeone et al 1990). In all cases similar morphological and phenotypical properties of neural-like cells were reported. More specifically, we have examined the neuronal development of a recently derived human EC cell line, TERA2.SP12. Upon the addition of retinoic acid to culture media, TERA2.SP12 EC cells have the capacity to differentiate and produce neural progenitors that ultimately express neurites (Przyborski 2001; Stewart 2003). These observations are consistent with data presented in this investigation. Here, when TERA2.SP12 EC cells were grown as monolayers and exposed to retinoic acid, approximately 10-20% of the cells appeared to follow the neural lineage. These neural like cells were observed to produce neurites after 4 weeks of incubation. Moreover, the nerve cells appeared to be sitting on top of a layer of non-neural cells, making this a heterogeneous culture. As mentioned above, such neural differentiation has been seen in other EC cell lines

in response to retinoic acid, including another sub-line of the TERA2 lineage, NTERA2.cl.D1 established by Andrews et al 1984. However, to date there has been little data concerning the identity of the non-neural cells found throughout the culture.

After introduction of HMBA into the culture medium, TERA2.SP12 EC cells also appeared to differentiate, but along an apparently different differentiation pathway. The cells within the culture stopped proliferation and changed morphologically forming a homogeneous culture containing cells with a large, flat oval phenotype, covering the entire culture area. These cells were non-neuronal and were morphologically similar to those non-neural cells found under the neurons in retinoic acid induced cultures. Similar HMBA-induced differentiation have also been observed in the NTERA2.cl.D1 EC cell line (Andrews et al 1990). Where it was noted that no significant neural differentiation occurred and the culture yielded a homogeneous layer of large, flat oval cells. The identity of such cells was not fully defined. HMBA as a differentiation-inducing agent has not been extensively studied in any other EC cell line.

Differentiation of TERA2.SP12 EC cells in response to retinoic acid and HMBA was confirmed by the down regulation of cell surface markers specific to undifferentiated stem cells. These markers, namely SSEA-3, SSEA-4 and TRA-1-60 have also been shown to be down-regulated in the human EC cell line 2102Ep after differentiation has been established (Andrews et al 1982). There was also a significant increase in the cell surface markers associated with differentiated cells in both retinoic acid and HMBA-induced cultures. The most significant increase was with EC cells induced to differentiate with retinoic acid. This could be due to the fact that two of the differentiation markers used, A2B5 and VINIS-56, are thought to be associated with the neural lineage (Eisenbarth et al 1979, Andrews et al 1996). The Me311 cell surface marker was not increased on any of the differentiated cultures. This observation was also consistent with the response by the NTERA2.cl.D1 EC cell line (Andrews et al 1990), indicating that the particular cell surface antigen that Me311

recognises is not affected by the differentiation of TERA2.SP12 EC cells induced by either retinoic acid or HMBA.

Together these observations indicate that the introduction of retinoic acid or HMBA into a culture of TERA2.SP12 EC cells induces cellular differentiation in a manner that is conserved with other sub-lines of the TERA2 lineage. It also appears that neuronal cell types are present in retinoic acid-induced cultures, but are absent in HMBA induced cultures, suggesting that the differentiation pathway in each case is different. However when looking only at the morphology of the cells, it appears that some similarities are retained between the two cultures. The non-neural cell types found in retinoic acid induced cultures appear very similar to those found throughout the entire culture of cells exposed to HMBA. This observation has not been previously reported.

When TERA2.SP12 EC cells were grown and differentiated as monolayer cultures it was observed that in retinoic acid induced cultures the expression of specific neural markers such as NF-68, NSE and β-tubulin-III occurred at high levels, whereas in HMBA induced cultures the expression was minimal, again indicating that retinoic acid monolayer cultures contain neurons whereas HMBA monolayer cultures do not. This expression correlated with the morphological appearance of neurons and agreed with the analysis of cell surface markers carried out by Andrews et al. 1990 in the NTERA2.cl.D1 human EC cell line. In contrast, both HMBA and retinoic acid induced cultures expressed non-neural markers namely keratin, cytokeratin-8, fibronectin and laminin, which along with other non-neural cell lineages, are expressed by epithelia cells. Cytokeratin-8 has been shown to be a good marker of early epidermal cell lines produced from all of the germ layers, including the ectoderm, where the development of skin epidermis occurs, showing expression of such cells up to day 12 of embryonic development. After this time point, only epidermal cells from the mesoderm and endoderm initiate positive reactions with this antibody, and skin epidermis from the ectoderm does not (Kemler et al 1981). Since EC cells have the ability to

differentiate into all three of the germ layers, it can be assumed that the expression levels seen with cytokeratin-8 in this instance could indicate the presence of an early epidermal lineage from either of these germ layers. This could include early epidermal lineages originating from the ectoderm, which is what this investigation is interested in. A pan-keratin marker also showed differential regulation. Both keratin markers revealed expression levels that were significantly higher in HMBA induced differentiated cells, suggesting that the introduction of HMBA into cultures of TERA2.SP12 EC cells may result in the differentiation of immature epidermal cell types. The expression of these markers is also seen at a lower level in retinoic acid induced cultures indicating that similar non-neural/epidermal cells may be present. However, from the preliminary data obtained in this study it can not be said that all of the non-neural differentiation that occurs in these cultures are of an epithelial/epidermal nature as other cell types have not been looked for, and the non-neural markers used are not exclusive to epidermal lineages. Therefore, to establish the nature of all of the non-neural cell-types present more investigation into this area is required. Fibronectin, an extra-cellular matrix protein, and laminin, a protein found in basement membranes can both be used as markers of the development of epidermal epithelia (Grover et al 1983). In this study both proteins were seen to be expressed in HMBA induced cultures and also at lower levels in retinoic acid induced cultures, providing further evidence that some of the non-neural cell types found in both cultures are possibly epithelial and with further investigation could be found to be epidermal in nature.

From these preliminary data it appears that when TERA2.SP12 EC cells are exposed to retinoic acid both neural and epithelial differentiation occurs whereas when the EC cells are exposed to HMBA it appears that only non-neural cells which could be epithelial differentiation results. This could indicate that during differentiation induced by HMBA a mechanism is present which inhibits neural differentiation. Two possible reasons for the lack of neurons found in HMBA cultures were considered. First, the default neural pathway

could be inhibited by the expression of anti-neural genes. Second, neural inhibition could occur by a mechanism similar to that found in the suppression of the default neural pathway modulated by BMP signalling during cell fate determination in the embryonic ectoderm. These hypotheses are discussed further below.

Some of the differentiation profiles shown to occur in the TERA2.SP12 EC cell line in this investigation have been reported in studies carried out on human ES cells. Initial work carried out by Schuldiner et al 2000 on the controlled differentiation of human ES cells discovered that the introduction of retinoic acid into culture medium up-regulates the expression of markers for neural differentiation (neurofilament heavy chain) and skin epidermal differentiation (keratin), similar to the expression profiles seen in this investigation with the introduction of retinoic acid. HMBA has not been used to induce differentiation of human ES cells, however other epidermal inducing factors such as BMP4 have been examined. In Schuldiner's study (2000) the introduction of BMP4 into culture medium resulted in the up-regulation of skin epidermal markers and an absence of neural markers, similar to the results obtained when TERA2.SP12 EC cells are induced to differentiate with HMBA. These findings add further basis to the argument that human EC cells represent a readily available, researchable model of human ES cells as previously reported (Andrews, 2001- book chapter reference). Reconstituted skin has been formed from murine embryonic stem cells (Coraux et al. 2003). These stem cells were grown on human normal fibroblasts as a feeder layer and exposed to either BMP-4 or ascorbic acid. After 8 days of incubation significant keratinocyte differentiation was recorded along with an increase of many epidermal markers. Coraux et al. went on to grow these keratinocytes on stainless-steel grids that allowed then to grow in a 3-dimentional environment thus stimulating the differentiation of reconstituted skin. These finding could be used as a way of expanding this study. TERA2.SP12 EC cells could be exposed to BMP-4 or ascorbic acid

under similar culture conditions and it could be noted if they would mimic this epidermal differentiation process.

4.2 Anti-neural Genes and the BMP Signalling Pathway Which May Block Neurogenesis During TERA2.SP12 EC Cell Differentiation with HMBA

The expression of anti-neural genes was investigated to deduce whether in HMBA cultures these genes could be suppressing the production of neural cell types. The anti-neural genes selected were transducin-like-enhancer of split 1 and 2 (*tle1* and *tle2).* Both genes are associated with the Notch signalling pathway that controls various cellular interactions involved in certain cell fate determinations (Liu et al 1996). Among the cell fates over which the pathway exerts some control are both epidermal and neural cell fates. Notch signalling appears to inhibit differentiation of stem cells until the correct morphogenetic signals are activated. *tle1* has been shown to be a negative regulator of postmitotic neuronal differentiation in the mammalian central nervous system once ectodermal patterning has taken place (Yao et al 2000). In this investigation *tle1* was seen to be expressed evenly throughout all samples tested suggesting that it is present in all cultures and it could therefore play some role in the suppression of neurogenesis and induction of epidermis. However further analysis will be required to confirm this, especially whether *tle1* is down-regulated during the formation of neurospheres from human EC cells since >95% of these cultures form exclusively neural cells (Stewart et al 2003). As noted above, the *tle2* gene is also associated with pathways controlling the differentiation of stem cells in neural or epidermal direction (Liu et al 1996) and has been shown to be expressed in epidermal progenitor cells. In the current study *tle2* expression was seen to increase as incubation with

either of the inducing agents continued which correlated with the presence of non-neural cell types that expressed markers of epithelial cells. These findings indicate that the production of epidermal lineages both in retinoic acid and HMBA induced differentiated cultures is likely. Again confirmation that this gene exerts a regulatory role would require further experimentation.

As cultures of TERA2.SP12 cells appear to be able to produce epithelial cells that express markers indicative of epidermal cell types and under different culture conditions appear to have an ability to produce neurons, it is proposed that differentiation by human TERA2.SP12 cells resembles development of the embryonic ectoderm. It is the ectoderm that gives rise to the formation of skin epidermis and the neural components of the CNS (reviewed in Weinstein and Hemmati-Brivanlou 1999 and in this investigation). The BMP signalling pathway appears to play a major role in the regulation of this ectodermal patterning during development (Wilson and Hemmati-Brivanlou 1995, Hawley et al 1995 and Massague 1998). To test whether TERA2.SP12 EC cells and their differentiated derivatives expressed the specific proteins and genes found in the BMP signalling pathway, expression levels of various genes was examined. In this study it was shown that *BMP4, BMP6, msx1, gata-1,* and *Smad 1,4,6,* and *7* were all expressed in cultures of both retinoic acid and HMBA-induced differentiated cells, both grown as aggregates and in monolayer cultures. This indicates that the BMP signalling pathway could be present and functional within these EC cells during differentiation, and that through this signalling the production of either neuronal or epidermal cells may be controlled. Interestingly, all of the proteins and genes tested showed even expression levels across all of the samples studied except for *gata-1*. This gene showed higher expression levels in aggregate cultures exposed to retinoic acid and correlated with high levels of epithelial markers. This could be due to the fact that *gata-1* is part of a transduction mechanism that induces epidermal differentiation by initiating the inhibition of the default neural differentiation pathway.

The occurrence of the BMP signalling pathway within other EC cell lines has been investigated. Caricasole et al 2000 carried out investigations in this area on the NTERA2.cl.D1 cell line. RT-PCR analysis from these EC cells showed high expression profiles of the co-SMAD, SMAD4, and BMP-specific SMAD transcripts within these cells, indicating that genes involved in the BMP signalling pathway were active. Given the data available on embryonic ectodermal patterning, Caricasole et al (2000) went on to hypothesise that the exposure of NTERA2.cl.D1 EC cells to BMPs would result in epidermal differentiation. The expression of a pan-keratin marker was studied, and they found it to be expressed at higher levels after the EC cells were exposed to BMPs, confirming that these non-neural cell types were indeed epidermal. Similar results have been obtained in this investigation, supporting our conclusion that TERA2.SP12 EC cells are capable of forming ectodermal derivatives as a possible consequence of BMP signalling.

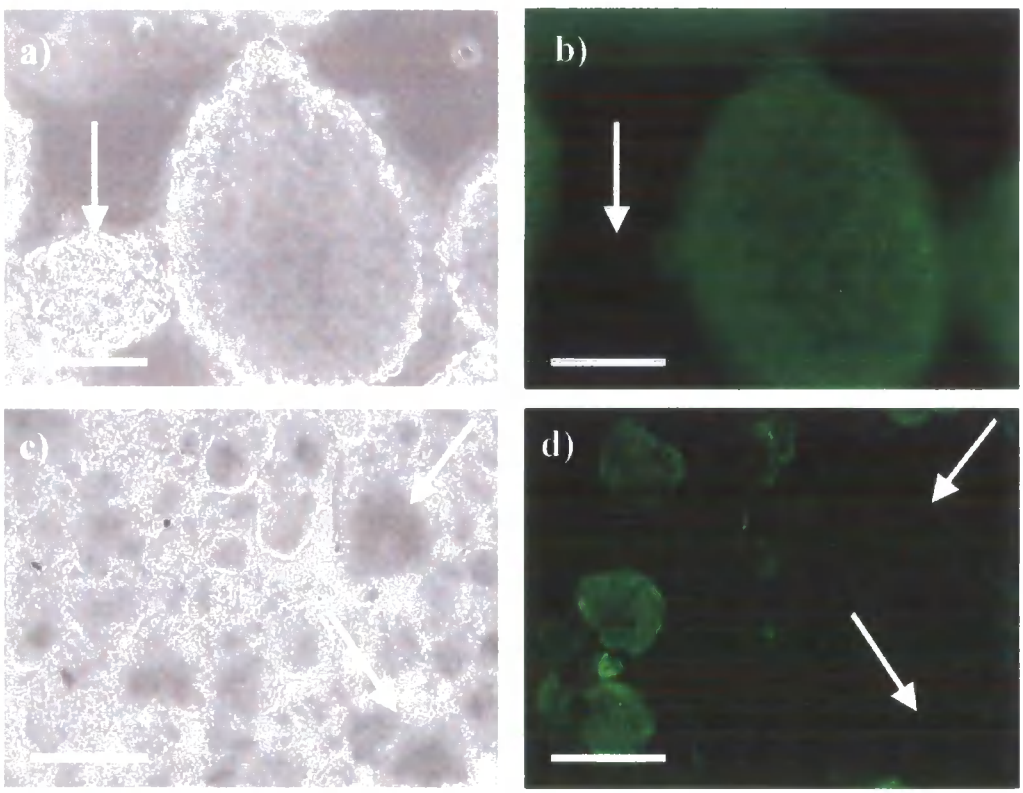
4.3 TERA2.SP12 Embryonal Carcinoma Cells Grown as Aggregates

The growth of TERA2.SP12 EC cells as aggregates in the presence of retinoic acid has been shown to greatly enhance the cellular differentiation of neurons (Stewart et al 2003). A possible explanation for this increased neuronal differentiation could be that as a result of this method of cell culture a disruption to the BMP signalling pathway occurs, blocking its ability to inhibit the default neural pathway. This hypothesis is currently under investigation in our laboratory and preliminary experimentation is included here.

Analysis of TERA2.SP12 aggregates grown in suspension in the presence of retinoic acid has been carried out. Aggregates formed in the presence of retinoic acid, termed neurospheres, are shown to contain >95% neurons following 14 days of incubation (Stewart et al 2003). Large neurospheres can be readily purified and form intricate neural networks when plated onto laminin coated surfaces. Live staining of developing suspended aggregates

has established that whilst most aggregate formations highly express neuronal differentiation markers such as VINIS-56, some smaller, irregular looking aggregates remain negative, and express cell surface markers specific for undifferentiated stem cells, such as TRA-1-60 (Horrocks unpub) (Figure 4.1). On-going investigations in our laboratory have identified these negative aggregates as being embryoid body-like structures. It is possible that these structures contributed toward the formation of non-neural epithelial cell types when plated onto coverslips. The further three weeks un-inhibited incubation of these cells on the coverslips may allow for the differentiation of large quantities of epithelial tissue from such embryoid body structures, although this would need to be verified. Studies carried out on F9 EC cell aggregates determined that when the cells were introduced to low concentrations of retinoic acid, the cells aggregated and formed embryoid bodies with an outer layer that appeared to represent a model of epithelium formation (Grover et al 1983). In this investigation, the early epidermal marker cytokeratin-8 appears to be expressed at a higher level in the retinoic acid aggregate cultures as opposed to HMBA aggregate cultures, which contrasts with observations from monolayer cultures. As suggested, a possible explanation for this increased expression of cytokeratin-8 is that when these aggregates are grown on coverslips for an extended period of time the undifferentiated cells found in the embryoid body-like structures undergo differentiation on the glass plate, and start to undergo epidermal layer formation as seen in the F9 model (Grover et al 1983). This may possibly explain the increased expression of epithelial markers in our retinoic acid culture system under the conditions tested. If this theory is correct then it may also explain the increased level of expression of the gene *gata-1* as mentioned above, since *gata-1* was also was expressed at a higher level in aggregates exposed to retinoic acid and can be used as an indication of epidermal differentiation.

FIGURE 4.1: ***Evidence of cell aggregates expressing stem cell markers in cultures exposed to retinoic acid.*** *Corresponding phase (a,c) and fluorescent images (b,d) of cells cultured for 11 days in suspension. Large, spherical aggregates expressed VINIS-56 whilst significantly smaller irregular aggregates (arrow), were negative for this marker (a,b). Conversely, irregular aggregates expressed the stem cell marker TRA-1-60, whilst larger regular spheroids did not (arrows). Bars: (a,b 150μ m), (c,d 400μ m). Images courtesy of G Horrocks.*



Results obtained from EC cells grown as aggregates and subsequently transferred to coverslips in the presence of either retinoic acid or HMBA also indicated that neural markers continued to be expressed at a high level in retinoic acid induced cultures but also appeared to be elevated slightly in HMBA samples compared with their monolayer culture counterparts. An explanation for this observation may involve cell-to-cell communication during differentiation and the disruption of the BMP signalling pathway. If cell-to-cell contacts are disrupted then the neural default pathway may be activated due to the absence of extracellular inhibiting factors, such as BMPs, therefore resulting in the production of neural cells within the culture. This has been shown to be the case in disassociation studies examining *Xenopus* embryonic ectoderm cells. When the dissociation assays were carried out on the ectoderm, cells that under normal circumstances would form epidermis, took on a neuronal cell fate (Grunz and Tacke 1989).

To summarise, the culture of aggregates in suspension greatly enhances neuronal differentiation (>95% Neurons) as previously described (Stewart et al 2003). However, Less than 5% of cells are therefore non-neuronal and have been shown to have an embryoid body-like structure. It is suggested here that this 5% of non-neuronal, undifferentiated cells must contribute to the further growth of epidermal cell types seen after the aggregates have been plated onto coverslips for a further three weeks. As there is a slight increase in neurogenesis in HMBA-induced cultures, it is also proposed that the lack of cell-to-cell contact consequential of this method of cell culture may potentiate any small amount of neural differentiation in the presence of HMBA

4.4 TERA2.SP12 Embryonal Carcinoma Stem Cells as a Model for Ectodermal Patterning in the Human Embryo

The data presented in this investigation provide some preliminary evidence which suggests that the TERA2.SP12 EC stem cell line could be a useful model viable for research into ectodermal patterning during human embryonic development. EC cells have already been proven to be a useful model of human development due to their close relationship with germ cells within the embryo (See Figure 1.5) (Andrews et al 2001). Every EC cell line appears to have a different capacity for differentiation. TERA2.SP12 EC cells have been shown to have a greater capacity for neural and glial formation (Przyborski 2001), and as shown in this study, also appears to have the capacity to differentiate into epithelial tissues indicative of immature epidermis.

Before the TERA2.SP12 EC cell line can be established as a model of human ectodermal patterning, further analysis will have to be carried out. Further clarification of the epidermal nature of the differentiated non-neural cells may involve the use of epidermal markers other than keratin, such as β1-integrin, E-cadherin and plakoglobulin, which are all markers of epidermal progenitors cells (Watt 1998). Further investigation is also required to establish the role of BMPs in the differentiation of cells into neural or epidermal cell types within this particular cell line. This would help verify whether the BMP signalling pathway is conserved between EC cell differentiation and embryonic ectodermal development.

If such further analysis indicated that the TERA2.SP12 EC cell line was a viable model for ectodermal patterning in the human then this would become a very useful research tool in many areas of science. For instance, as a model for use in drug discovery programmes and toxicological testing, perhaps ultimately leading to the production of synthesized skin grafts, and neural transplantations to overcome tissue trauma and degenerative disease. Furthermore, a model of ectodermal development EC cells would also be useful as a tool for

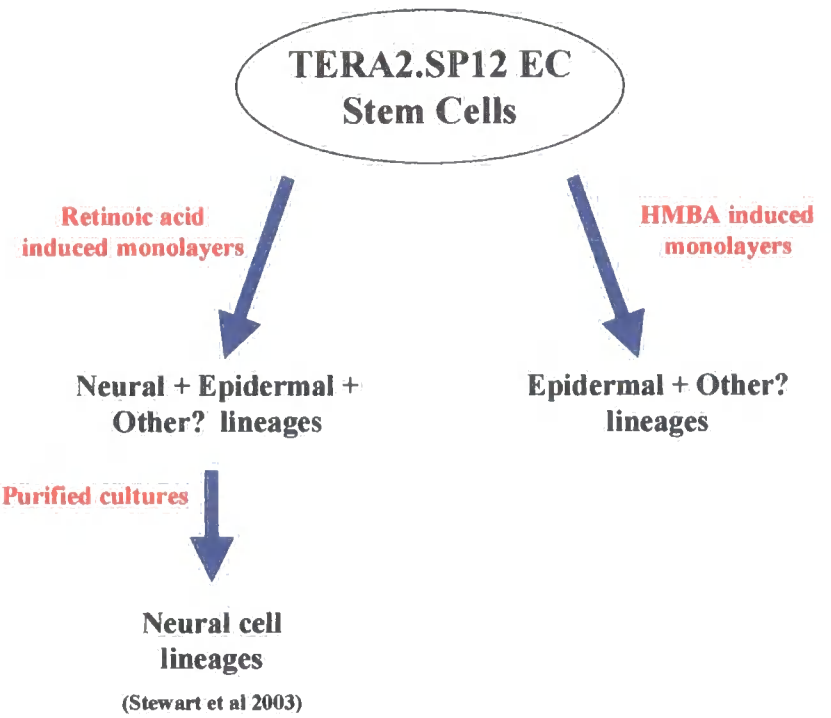
understanding the molecular mechanisms underlying human ectodermal development. At present, there is little data available on human embryonic development due to the many ethical and moral issues surrounding the use of embryonic and foetal tissues required for this type of research. Since the TERA2.SP12 cell line originates from unwanted tumour tissue it would overcome these particular ethical and moral issues.

For the areas of research outlined, human EC cells appear to form a more robust, readily available and more user-friendly cell line to work with than human ES cells. Although human ES cells are derived from the blastocysts and would ideally serve as a better model, they are still surrounded by strict guidelines for their use and there are practical and technical problems associated with their handling such as their dependence on feeder cells and their tendency to spontaneously differentiate (Andrews et al 2001).

CONCLUSION

The differentiation pathways of TERA2.SP12 EC stem cells are different when exposed to either retinoic acid or HMBA. The introduction of retinoic acid into the culture medium initiates the differentiation of neural and epithelial sub-types, whereas the introduction of HMBA initiates the almost exclusive differentiation of epithelial cells (Figure 4.2). The pathways appear to bear a relationship to the differentiation pathways seen during ectodermal patterning in the developing vertebrate embryo. I conclude therefore, that the human TERA2.SP12 EC stem cell system may be a viable and practical model for the use of research into this area of developmental biology.

FIGURE 4.2 ***Ectodermal patterning by human embryonal carcinoma cells.*** *Schematic illustrating the primary directions of TERA2.SP12 cell differentiation studied during this investigation.*



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Web Pages:

- http://www.kumc.edu/instruction/medicine/pathology/ed/ch_18/c18_dermoid_gross.html
- http://www-medlib.med.utah.edu/WebPath/PEDHTML/PED050.html